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13. ABSTRACT (Maximum 200 Words)

We proposed to apply two novel technologies to the development of an approach suitable for screening for ovarian cancer in high and low risk women. The first of these is a novel approach to the development of antibodies which will recognize specific phospholipids and lysophospholipids present in ovarian cancer patients and the second of these is SELDI tof mass spectroscopy. These two technologies will be merged with powerful computing tools to develop approaches capable of detecting ovarian cancer at an early, curable stage. This approach will further benefit from the expertise of the Mills laboratory (LPA screening, SELDI tof) with that of the Prestwich laboratory (lipid synthesis and antibody development).

Progress

We have demonstrated that SELDI mass spectroscopy has the ability to detect model lysophospholipids present in plasma and serum. The sensitivity of the assay using standard capture chips is low and would require relatively large volumes of sera to detect the different lysophospholipid isoforms. This will require prepurification approaches to concentrate the lipids which we have now developed. We have obtained a pan sphingosine 1 phosphate antibody as a capture reagent. This antibody is able to bind all isoforms of S1P and when combined with SELDI should allow detection of isoforms of this lysophospholipid. We have shown that the antibodie can detect S1P levels in complex ascites mixtures. We are currently evaluating different CHIP forms for SELDI and developing LPA antibodies as well as obtaining LPA binding proteins to increase the ability to capture lysophospholipids through collaborative approaches.

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INTRODUCTION

Background and Preliminary Data

Ovarian cancer remains the 5th most frequent cause of death from cancer in women. Indeed, the current cure rate for ovarian cancer is under 40%, not substantially different from that in the 1950s. This abysmal prognosis occurs, in most part, due to ovarian cancer being diagnosed at a late stage where current therapies are ineffective. Unfortunately more than 75% of patients are diagnosed when the disease has spread beyond the pelvis. Thus a method to detect ovarian cancer at an early curable stage has the potential to provide an immediate and major impact on this devastating disease. This is particularly important for individuals at high risk either because of a strong family history or proven abnormalities in BRCA1 or BRCA2.

The identification of secreted molecules, which contribute to the pathophysiology of ovarian cancer, provides a major opportunity to identify markers that could contribute to early diagnosis. We have demonstrated that the most potent growth factor activity in ascites of ovarian cancer patients consists of multiple forms of lysophosphatidic acid (LPA). LPA increases proliferation, prevents apoptosis and anoikis, increases invasiveness, decreases sensitivity to cisplatin (the most effective drug in ovarian cancer), and increases production and activity of multiple growth factors, proteases and mediators of angiogenesis. Thus LPA contributes to the pathophysiology of ovarian cancer. We and others have subsequently demonstrated that multiple additional bioactive lysophospholipids, including lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), sphingosine 1 phosphate (S1P) and lysophosphatidylserine (LPS) exhibit pleiomorphic effects on ovarian cancer cells.

Ascites from ovarian cancer patients contains high levels of lysophospholipids including multiple forms of LPA, lysophosphatidylinositol (LPI), LPC, SPC and S1P. Reports from our and other laboratories indicate that plasma and sera from ovarian cancer patients contain aberrant levels of these lysophospholipids. This suggests that an efficient method to determine levels of lysophopholipids in serum or plasma could provide an effective method to screen for ovarian cancer.

Over 500 species of lysophospholipids are present in plasma and serum. Currently, lysophospholipids are quantified by lipid extraction of a serum sample followed by Mass spectrometry (MS). While MS analysis is highly accurate, the methodology has limited throughput for large-scale screening of patient samples. Further, the need to use organic solvents to prepare specimens for MS limits the quantitation and the applicability of the method. Surface-enhanced laser desorption and ionization time of flight (SELDI tof) mass spectroscopy analyses of "markers" diagnostic of ovarian cancer have identified a number of candidates with mass/charge ratios of under1000, suggestive that these could be lysophospholipids rather than peptides. Identification of these markers required the global unbiased analysis of many different molecules in serum combined with powerful algorithms designed to identify patterns indicative of the presence of cancer.

We propose to apply two novel technologies to the development of a high through-put technology suitable for screening for ovarian cancer in high and low risk

women. The first of these is a novel approach to the development of antibodies to specific phospholipids and lysophospholipids and the second of these is SELDI tof mass spectroscopy. These two technologies will be merged with powerful bioinformatics tools to develop heuristic algorithms capable of detecting ovarian cancer at an early, curable stage. This approach will benefit from the expertise of the Mills laboratory (LPA screening, SELDI tof) with that of the Prestwich laboratory (lipid synthesis and antibody development).

To develop efficient methods for analysis of lysophospholipids in ovarian cancer patients, we will:

1. **Assess the efficacy of novel LPA/PA lipid antibodies developed by our group in capture and analysis of LPA/PA directly in serum and plasma using SELDI-tof**
2. **Determine whether non-specific matrices (hydrophobic C16, anionic SAX2) can be used to directly determine phospholipid and lysophospholipid levels using SELDI-tof**
3. **Develop additional anti lysophospholipid antibodies and determine their utility in analysis in Seldi-tof**

Significance: Over 75% of ovarian cancer patients are diagnosed when the disease has spread beyond the pelvis. At this stage of disease, the cure rate is under 15%. This is in contrast to the cure rate for early stage ovarian cancer, which can approach 90%. Thus any approach that can allow diagnosis of ovarian cancer at an earlier curable stage has the potential to have a marked impact on this devastating disease.

BODY

Statement of Work

Task #1 Assess the efficacy of novel LPA/PA lipid antibodies developed by our group in capture and analysis of LPA/PA directly in serum and plasma using SELDI-tof (months 1-24)

This specific aim was dependent on the production of anti-lipid antibodies and identification of LPA and PA binding proteins for selective capture of these ligands from serum and ascites. Recently, we have experienced difficulty in achieving selective lipid binding by anti-lipid antibodies, scale-up production issues, and problems in obtaining homogenous, healthy anti-lipid producing clones using the approaches proposed in the application. As noted below however we have been working with Roger Sabadini at UCSD with an anti S1P antibody. We have now adapted the approach that he used to develop the anti-S1P antibody and are now developing anti LPA antibodies with this approach.

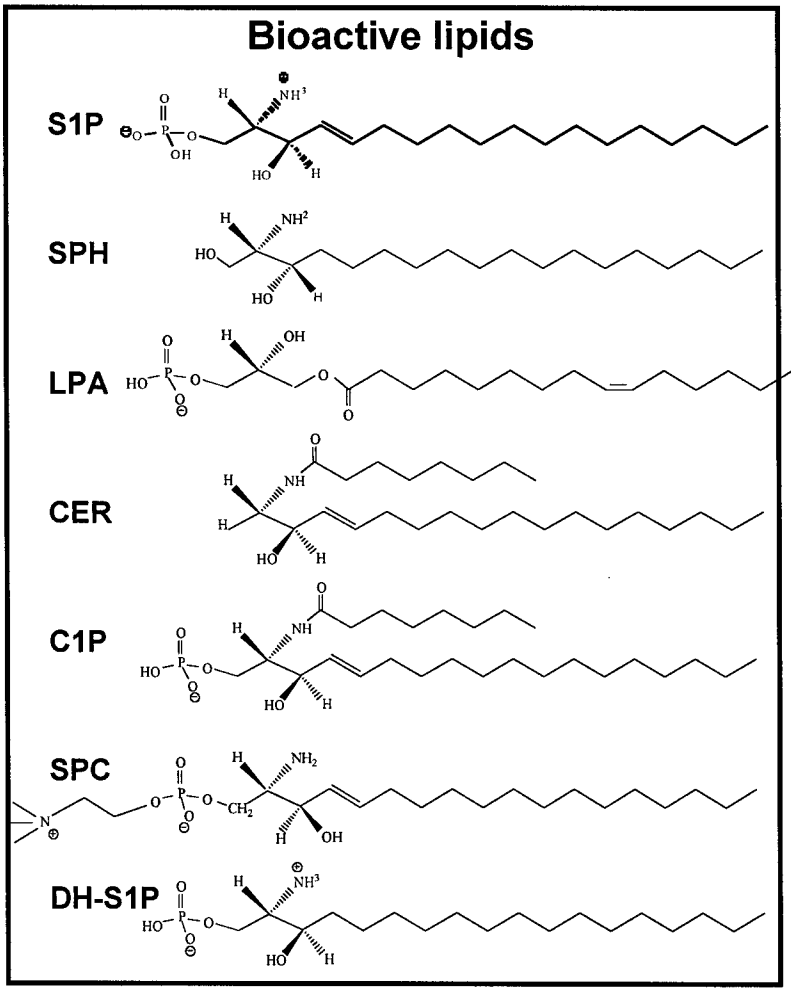
Last year we broadened the initial aim to include discovery of additional lysophospholipid and lipid binding proteins that could be readily produced as recombinant proteins and engineered to have particular useful lipid binding characteristics. To this, end, we have employed our tethered LPA and PA reagents, used earlier for ant lipid antibody elicitation, to make affinity resins for identification of novel LPA binding proteins from fibroblasts and cancer cells. The same modifications are in process for LPC, PC, LPI, and PI lipids. To identify new lysophospholipid binding proteins, a MS proteomics collaboration was established with Drs. J. Gettemans and J. Vanderkerckhove at the University of Ghent (Belgium). This collaboration has the benefit of discovering novel proteins in the LPA signaling pathway, which are likely to be important as diagnostic markers in their own right, as well as being important reagents for the proposed SELDI-MS capture method. The collaboration has been broadened with other lysophospholipids as the reagents have become available. The engineering of new specificities by mutagenesis will be incorporated later in this program, following identification of scaffold lipid-binding proteins and determination of their 3D structures.

While we have been preparing affinity matrices and antibodies, we have prepared a large number of LPA analogs. These LPA analogs will serve a control in the assays and also as markers for the analysis. Thus once the antibodies or affinity reagents are identified, we will be able to move this forward much more quickly (note the modified time lines).

Although, there has been difficulty in development of sufficient amounts of LPA/PA antibodies for this aim, we have obtained a high affinity pan S1P antibody from Dr. Roger Sabadinni at UCSD. This antibody binds all forms of S1P and demonstrates efficacy in determining S1P levels using ELIZA. Our recent data indicates that there are

only one major form of S1P in ovarian cancer patients. We have thus proceeded with the anti S1P antibodies as a first analysis.

Figure 1



As can be seen from the competitive ELISA, the anti-S1P antibody interacts with S1P and dihydroS1P and modestly with SPC. It does not interact with other lysophospholipids. Given that the concentrations of SPC and dihydroS1P in plasma are low, the ELISA reliably measures S1P levels.

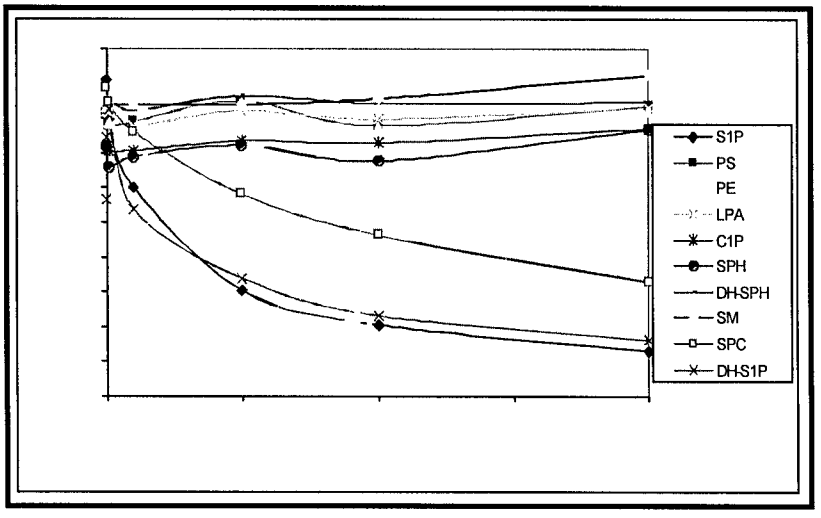
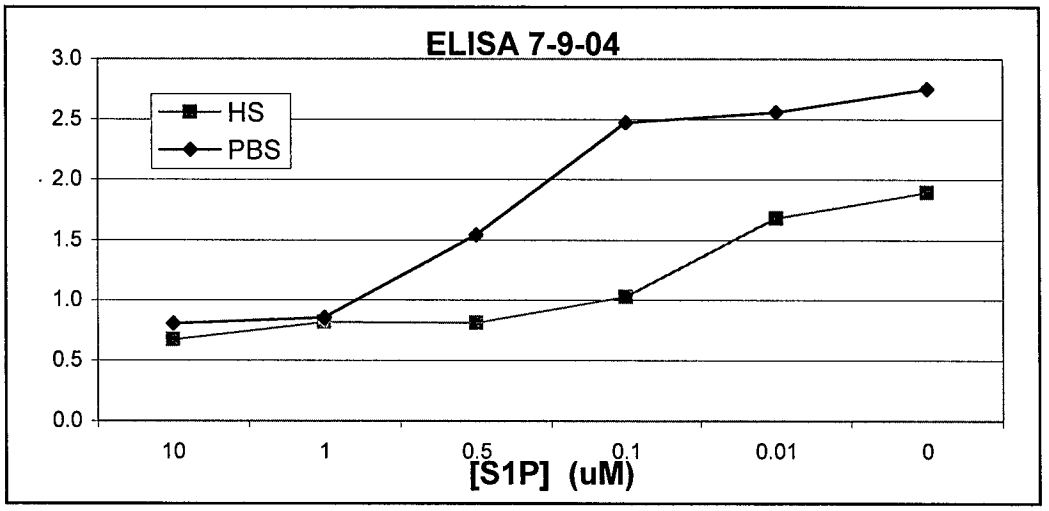


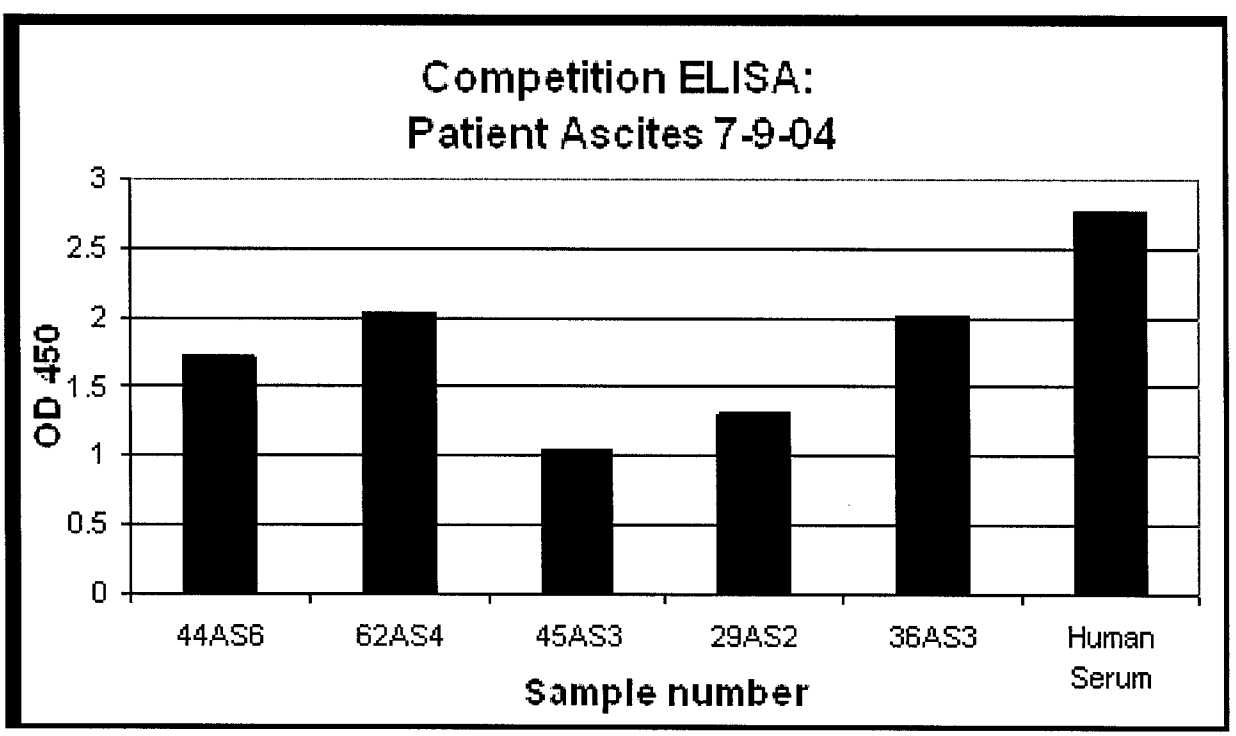
Figure 2

Figure 3



Above is a competition ELISA with known amounts of S1P demonstrating the linearity of the curve in phosphate buffered serum or human serum.

Figure 4



This is a competition ELISA with patient ascites samples as an example. Lower values represent higher amounts of S1P. Thus all of the model samples were elevated compared to human serum arguing that the approach will be able to detect and quantify S1P in serum and in ascites.

Either the LPA binding protein or LPA antibody approaches are expected to be successful. We will proceed with the tasks listed herein with the S1P antibodies. Note the shortened time lines from the original application based on current progress.

1.1 Couple antibodies to Seldi-tof matrices (Months 1-4)

1.2 Validate Seldi tof analysis with model lysophospholipids/phospholipids (Month 5)

1.3 Validate Seldi tof analysis with sera and plasma with known lipid composition (Months 6-8)

1.4 Validate quantification of Seldi tof analysis with stable isotope labeled lysophospholipid/lipid spiked into plasma and serum (Months 8-10)

1.5 Validate Seldi tof analysis with teaching and training set of serum and plasma samples from ovarian cancer patients (Months 10-12)

Each of the subsequent steps in this Task will be dependent on the successful completion of the preceding Task

Task #2 Determine whether non-specific matrices (hydrophobic C16, anionic SAX2) can be used to directly determine phospholipid and lysophospholipid levels using SELDI-tof

We have made significant process in this task. We have applied model lysophospholipids to matrices and demonstrated an ability to detect the model lysophospholipids in sera. We can readily detect 0.2nmol of LPA and other lysophospholipid isoforms in a single spot using the SELDI matrix. Total LPA levels in plasma, sera and ascites are between 100nM and 80μM. These are readily detectable requiring a maximum of 2ml of plasma, and very low amounts of sera and ascites. However, in order to detect isoforms of LPA that may be present at much lower concentrations, we would need however as much as 7ml of plasma loaded on to the current matrix. Thus we are now assessing different matrices and washing conditions. Nevertheless, 7 ml of plasma could be obtained from patients for analysis. This will require a pre purification step before loading onto the Seldi matrix. This is currently in progress.

We have proceeded with prepurification approaches and have identified a number of approaches that work well to produce LPA from fetal bovine serum. We have completed the IRB requirements and are proceeding with the studies as indicated below. Once again we have been able to compress the time lines due to the preliminary studies that have been done.

PA and LPC are present at much higher levels than LPA, allowing ready detection. LPI and LPE are present at similar levels to LPA suggesting that the approach is on target and will be able to detect the multiple different lipids present in plasma, sera and ascites.

This year Dr Tanaka and colleagues (Tanaka et al 2004) demonstrated that a Maldi time of flight mass spec approach similar to that proposed in this application can detect LPA in egg white and further that it could be used to detect material in blood. The approach used a phosphate affinity matrices and a mass shift associated with a zinc complex. However, just as we have noted in the first report, the approach requires prepurification of LPA from the complex mixture in order to apply enough to the plate. The ability to detect LPA and also to determine the fatty acid chain mixture is compatible with an approach to make this an efficient method to detect and quantify LPA. The ability to quantify and characterize the various species of LPA showed acceptable CVs for development of a clinical grade assay.

The detection approach of cocrystalizing LPA and Zn is an improvement of the approach proposed here and will be implemented in our ongoing studies.

We have adopted a similar extraction approach using calf serum as a control. Calf serum is mixed with chloroform, methanol KCL and ammonia hydroxide and allowed to phase separate. The LPA is moved from one phase to another using pH 2 HCL.

2.1 Identify and obtain matrices for analysis (months 1-4)

2.2 Determine conditions for binding of lysophospholipids/phospholipids (months 5-6)

2.3 Determine conditions for washing of lysophospholipids/phospholipids (months 5-6)

2.4 Select affinity matrix for further analysis (month 7)

2.5 Validate Seldi tof analysis with model lysophospholipids/phospholipids (Month 8)

2.6 Validate Seldi tof analysis with sera and plasma with known lipid composition (Months 9)

2.7 Validate quantification of Seldi tof analysis with stable isotope labeled lysophopholipids/phospholipids spiked into plasma and serum (Months 10)

2.8 Validate Seldi tof analysis with teaching and training set of serum and plasma samples from ovarian cancer patients (Months 11-12)

Task #3 Develop additional anti-lysophospholipid/lipid antibodies and determine their utility in analysis in Seldi-tof

As noted above, based on the progress described above, this task has been incorporated into Task#1.

The laboratory member working on these projects left the laboratory. A new PDF Mandi Murph has been hired and will take over the studies in the next few weeks.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that model lysophospholipids can be detected at concentrations present in patients by SELDI tof
2. Developed methods to prepurify lipids for attachment to SELDI plates
3. Obtained sufficient quantities of a high affinity S1P antibody for assessment by SELDI tof
4. Demonstrated specificity of the anti S1P antibody
5. Demonstrated ability to quantitate S1P by competitive ELISA
6. Synthesized and characterized a large set of LPA analogs
7. Initiated identification of high affinity lysophospholipid binding proteins.

REPORTABLE OUTCOMES

None

CONCLUSIONS

Overall, there has been significant progress in achieving the aims of the proposal. We have obtained a high affinity S1P antibody and are linking it to appropriate matrices. We have demonstrated utility of the anti S1P antibody in competition ELISA assays. We have developed approaches to develop anti-LPA antibodies with Dr. Sabadini. We have demonstrated that Seldi Tof has the ability to detect the amounts of lysophospholipids present in plasma, sera and ascites. The proposed improvements in the approach should increase the sensitivity of the assay to allow the detection of different isoforms in plasma. Increased sensitivity is not required for sera or ascites.

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APPENDICES

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Lysophosphatidic Acid Production and Action: Validated Targets in Cancer?

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Abstract The completion of the human genome project, the evolution of transcriptional profiling and the emergence of proteomics have focused attention on these areas in the pathophysiology and therapy of cancer. The role of lysophospholipids as potential mediators in cancer pathophysiology, screening and management has taken a major leap forward with the recent cloning of several enzymes involved in the metabolism of lysophospholipids. Lysophospholipids, although small molecules, contain a high "informational" content. Differences include the nature of the phosphate head group, the regiochemistry of the fatty acyl chain on the glyceryl backbone, the presence of ether versus ester linkages to the backbone, and the length and saturation of the fatty acyl or alkyl chain. This informational content is sufficient to result in a marked structure function activity relationship at their cognate receptors. Thus the emerging discipline of "functional lipidomics" is likely to prove as important as genomics and proteomics in terms of early diagnosis, prognosis, and therapy. Lysophospholipid levels are elevated in vivo in a number of pathophysiological states including ascitic fluid from ovarian cancer patients indicating a role in the pathophysiology of this devastating disease. Although controversial, levels of specific lysophospholipids may be altered in the blood of cancer patients providing a potential mechanism for early diagnosis. Several of the enzymes involved in the metabolism of lysophospholipids are aberrant in ovarian and other cancers. Further, the enzymes are active in the interstitial space, rendering them readily accessible to the effects of inhibitors including antibodies, proteins, and small molecules. In support of a role for lysophospholipids in the pathophysiology of cancer, expression of receptors for lysophospholipids is also aberrant in cancer cells from multiple different lineages. All of the cell surface receptors for lysophospholipids belong to the G protein coupled receptor family. As over 40% of all drugs in current use target this family of receptors, lysophospholipid receptors are highly "druggable." Indeed, a number of highly specific agonists and antagonists of lysophospholipid receptors have been identified. A number are in preclinical evaluation as therapeutics. We look forward to the next several years when the role of lysophospholipids in physiology and the pathophysiology and management of cancer and other diseases are fully elucidated. J. Cell. Biochem. 92: 1115–1140, 2004.

Key words: lysophosphatidic acid; autotaxin; cancer; treatment

Overview

Lysophosphatidic acid, the simplest phospholipid, exhibits a broad spectrum of activity in many different cellular lineages and contributes to multiple physiological and likely to many pathophysiological processes in vivo [see Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003 for reviews and

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elsewhere in this issue]. LPA mediates it effects by binding to G protein coupled receptors with subsequent activation of heterotrimeric G proteins and downstream events including increases in cytosolic calcium, alterations in cAMP levels, Rac and Rho small GTPases, activation of phospholipase C, protein kinase C, the phosphatidylinositol 3 kinase pathway, the RAS MAPK pathway, proteases and, by as yet somewhat unclear mechanisms, tyrosine kinases [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. This latter effect is likely due, in part, to the activation of proteases and release of cell surface growth factors with subsequent activation of the epidermal growth factor receptor family [Prenzel et al., 1999; Gschwind et al., 2003]. There are four different LPA receptors so far characterized on the surface of mammalian cells, three members of the Edg family of receptors (LPA1, 2, 3 previously known as Edg 2, 4, 7, respectively), and a newly identified receptor LPA4 (previously GPR23/P2Y9) of the purinergic receptor family [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Noguchi et al., 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. Through activation of these GPCR and subsequent downstream signaling, LPA induces cellular proliferation, cellular differentiation, regulates cell–cell interactions, inhibits cell death, increases cellular motility, increases invasiveness, increases the production of cytokines including those affecting formation and maintenance of new vessels, and increases the production and action of proteases [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002b; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. On an organism level, LPA is implicated in complex physiological states such as immunological competence, brain development, wound healing, coagulation, and regulation of blood pressure [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. LPA has recently been implicated as a physiological ligand for PPARγ, a member of the nuclear hormone receptor superfamily [McIntyre et al., 2002]. Recently, LPA was shown to induce neointima formation—a prelude to atherosclerosis in humans—by interaction with PPARγ in a rat carotid artery model [Zhang et al., 2004]. Importantly, LPA analogues that were inactive at the GPCRs were potent activators of PPARγ in this assay [Xu and Prestwich, 2002], demonstrating that LPA acting on this nuclear transcription factor was both necessary and sufficient for neointima formation.

As the effects of LPA are pleiomorphic and could potentially contribute to the development of multiple pathophysiological states, under normal circumstances the production and degradation of LPA is in a tight equilibrium with normal levels of LPA being in the 100–200 nM range or lower [Xu et al., 1998; Xiao et al., 2000; Shen et al., 2001; Aoki et al., 2002; Baker et al., 2002; Sano et al., 2002]. Levels of LPA are elevated during wound healing and in a number of physiological media such as saliva and ovarian cyst fluid [Westermann et al., 1998; Sugiura et al., 2002]. The physiological functions of LPA suggest that LPA could contribute to a number of pathophysiological states including cancer, autoimmune or immunodeficiency disease, atherosclerosis, and ischemia reperfusion injury [Fang et al., 2002; Graler and Goetzl, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Okusa et al., 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. Indeed as described above, the physiological responses to LPA parallel events, which must occur for the full expression of the malignant phenotype including the ability to invade and metastasize [Hanahan and Weinberg, 2000; Fidler, 2003]. Although the underlying mechanisms remain elusive, aberrations in production and degradation of LPA have been identified in cancer cells as well as in cancer patients, in particular in the markedly elevated levels of LPA in the ascitic fluid of ovarian cancer patients and in autocrine activation loops in ovarian and prostate cancer [Shen et al., 1998; Eder et al., 2000; Fang et al., 2002; Mills et al., 2002; Xie et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003; Sengupta et al., 2003]. In combination with alterations in LPA receptor expression and potentially receptor function in multiple cancer

lineages, this implicates LPA as an important mediator in the pathophysiology of cancer [Goetzl et al., 1999; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003]. As a corollary, LPA production and degradation, receptors and signaling are high quality targets for therapy [Feng et al., 2003; Mills and Moolenaar, 2003].

LPA Production

LPA is a critical component of the production and remodeling of lipids that occurs intracellularly. Both calcium-independent phospholipase A2 (iPLA2) and calcium-dependent PLA2 (cPLA2) contribute to this process intracellularly (Fig. 1). Intracellular LPA can activate the PPARγ receptor, however, the physiologic role of this process remains to be clarified [McIntyre et al., 2002; Zhang et al., 2004]. Through conversion to PA, LPA also regulates membrane curvature and formation of caveoli, which are critical to internalization and sorting of signaling complexes [Schmidt et al., 1999; Kooijman et al., 2003]. The unconventional lipid lysobisphosphatidic acid (LBPA) has recently been shown to induce the formation of multivesicular liposomes in vitro, and, with a protein partner Alix, the organization of endosomes in vivo [Matsuo et al., 2004]. Thus, by activating intracellular receptors and by serving as a substrate for enzymes such as endophilin and LCAT, LPA plays an important role in cellular functions (Fig. 1). Whether, under physiological conditions, extracellular LPA migrates to the cytosol in sufficient concentrations to contribute to these processes or whether intracellularly produced LPA is the only relevant source remains to be determined. Further, it is not clear that intracellular LPA is exported from the cell to participate in intercellular signaling. Indeed a series of secreted and ectoenzymes with their catalytic surface outside the cell appear critical to the production and metabolism of extracellular LPA.

As noted above, under physiological conditions, plasma LPA levels are maintained at low concentrations in the range of 100–200 nM [Xu et al., 1998; Xiao et al., 2000; Shen et al., 2001; Aoki et al., 2002; Baker et al., 2002; Sano et al., 2002]. LPA levels in plasma represent the steady state attained by separately regulated rates of production, degradation, and clearance. The removal of LPA from the bloodstream is extremely rapid, suggesting that degradation or clearance is very efficient. In addition to containing low levels of LPA, inhibitory factors in plasma limit LPA activity and production [Sano et al., 2002]. Thus the bioavailable levels of LPA in plasma may be below those able to optimally activate LPA receptors. Nevertheless, plasma contains both the enzymes and the substrates required for LPA production [Aoki et al., 2002; Sano et al., 2002]. Incubation of plasma at 37°C, e.g., results in an increase in LPC levels and a concomitant LPA increase resulting in the production of micromolar amounts of LPA [Aoki et al., 2002].

Cellular activation such as occurs during blood clotting, wound healing, or inflammation increases local production and action of LPA. Serum, in comparison to plasma, contains much higher levels of LPA with levels being between 1 and 5 μm [Aoki et al., 2002; Sano et al., 2002]. In tissue culture, it is likely that continuous production of LPC and LPA results in regeneration of LPA over time. Indeed, the regeneration of LPA, a potent growth and survival factor likely accounts for the quality of serum as a growth media. Platelets were initially proposed to be the main source of LPA in serum as they can produce LPA on activation [Gerrard and Robinson, 1989]. However, the amount of LPA released by activated platelets is insufficient to explain the increase in LPA levels that occur on blood clotting and likely accounts for only a small portion of the LPA present in serum [Aoki et al., 2002; Sano et al., 2002]. Further the fatty acyl chain composition of LPA produced by platelets [Gerrard and Robinson, 1989] 16:0 > 18:0 > 20:4 > 18:1 > 18:2 is markedly different from that present in plasma (18:2 > 18:1 = 18:0 > 16:0 > 20:4) or serum (20:4 > 18:2 > 16:0 = 18:1 > 18:0) [Sano et al., 2002]. This suggests that different molecular processes regulate production or degradation of LPA by platelets and the production and degradation of LPA that occurs in plasma or during the coagulation process that produces serum. LPA1 and -2 demonstrate selectivity towards saturated LPA as ligands, whereas LPA3 and -4 preferentially bind unsaturated versions of LPA [Bandoh et al., 1999; Yang et al., 2002a; Noguchi et al., 2003; Tigyi and Parrill, 2003]. Thus the different forms of LPA produced by platelets and during coagulation could activate different species of LPA receptors resulting in differential functions.

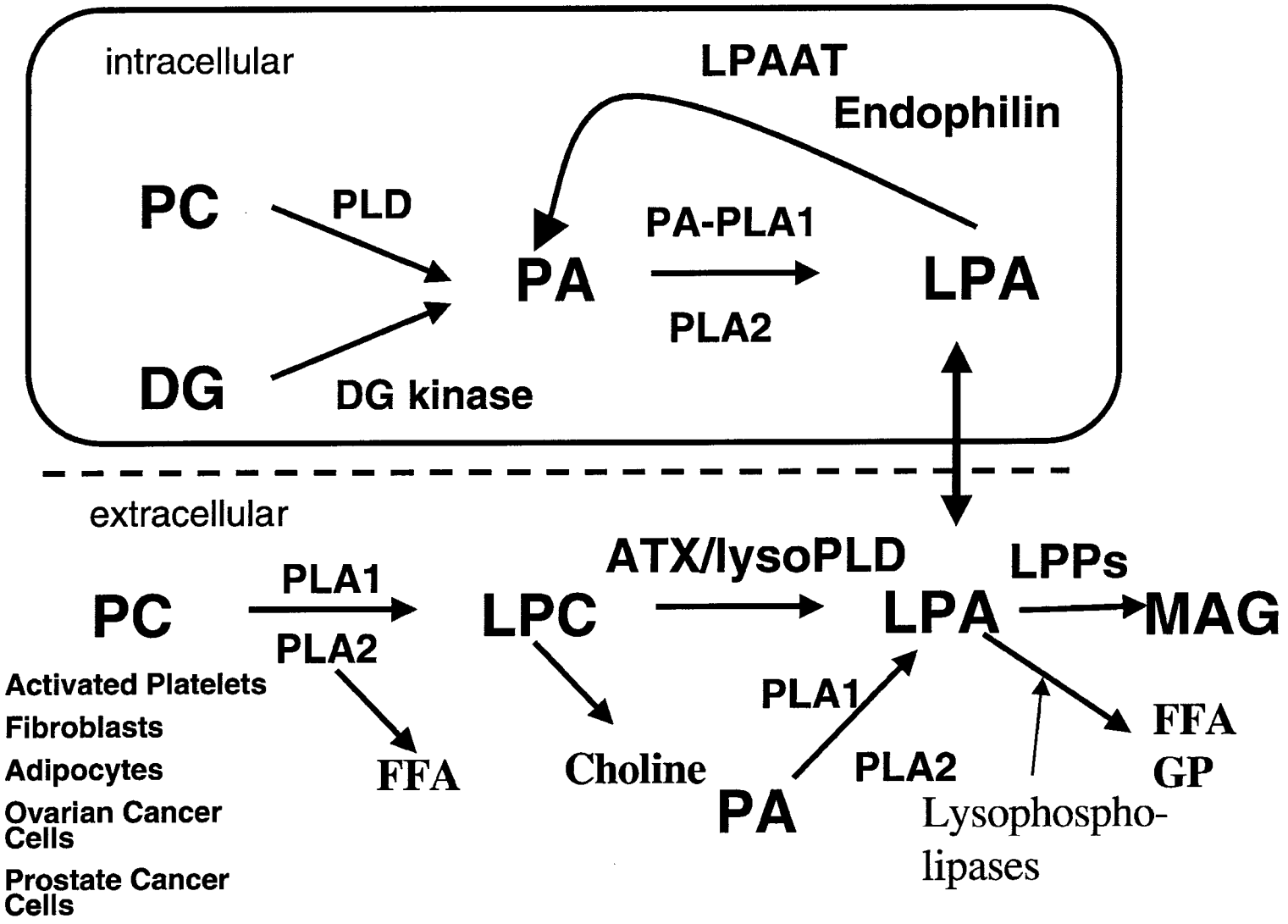


Fig. 1. LPA metabolism pathways the major intracellular and extracellular pathways of production and degradation of LPA are outlined. Whether intracellular and extracellular LPA are independent pools or whether there is exchange between these pools is unknown. Intracellular and extracellular LPA mediate different but important functions that could contribute to the pathophysiology of cancer. Cells known to produce LPA are indicated at the lower left.

Platelets contain high levels of phospholipases capable of cleaving phospholipids to produce lysophospholipids [Aoki et al., 2002; Sano et al., 2002]. Group IIA secretory phospholipase A2 (sPLA2, pancreatic) and phosphatidylserine-specific phospholipase A1 (PS-PLA1) are released from platelets activated by thrombin or calcium ionophores and likely contribute to the production of LPA by producing lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), and lysophosphatidylserine (LPS) precursor molecules [Aoki et al., 2002; Sano et al., 2002]. PS-PLA1 produces *sn*-2 lysophospholipids with unsaturated fatty acyl chains. At either high or low pH, the *sn*-2 fatty acyl chain migrates to the *sn*-1 site with equilibrium favoring *sn*-1 lysophospholipids, however, at neutral pH *sn*-2 lysophospholipids are relatively stable. As noted above, these forms of LPA may selectively activate LPA3 and -4 [Bandoh et al., 1999; Noguchi et al., 2003]. In addition to sPLA2 IIA, it is also likely that several other isoforms of sPLA2 can contribute to LPA production following platelet activation or in serum as expression of sPLA2 isoforms in platelets is species dependent and also LPA production is not altered in mice genetically deficient in group IIA sPLA2 [Fourcade et al., 1998].

sPLA2 has limited ability to hydrolyze lipids in intact cell membranes [Kudo et al., 1993; Fourcade et al., 1995, 1998]. sPLA2 selectively hydrolyzes lipids present in damaged membranes, membranes of activated cells or microvesicles such as those released during apoptosis or produced by cancer cells to produce *sn*-1 lysophospholipids [Kudo et al., 1993; Fourcade et al., 1995, 1998]. Disruption of the membrane microenvironment by phosphatidic acid (PA) produced by the action of phospholipase (PLD) or activation of intracellular signaling pathways such as thrombin-induced increases in cytosolic calcium render cellular membranes more susceptible to the action of sPLA2 [Kudo et al., 1993; Fourcade et al., 1995, 1998; Kinkaid et al., 1998]. PLD also induces the production of extracellular microvesicles that are susceptible to the effects of sPLA2 [Morgan et al., 1997]. Strikingly, LPA induces the activation of PLD, increases cytosolic free calcium and increases PA levels compatible with LPA contributing to its own production [van der Bend et al., 1992; Kam and Exton, 2004]. The microenvironment wherein PS-PLA1 can hydrolyze lipids is not clear but it may also require access to substrates in particular structures limiting the production of LPA in plasma. Taken together, this suggests that LPA production likely occurs at sites of cellular injury or disruption. Further as tumors exhibit high levels of spontaneous apoptosis that produces vesicles, production of cellular vesicles and aberrations in membrane composition and symmetry [Fourcade et al., 1995; Ginestra et al., 1999; Andre, 2002], LPA may be produced at elevated levels in the tumor microenvironment.

In comparison to LPA, plasma contains high levels of phospholipids and lysophospholipids [LPC levels exceeding 100 μM and phosphatidylcholine (PC) levels approaching 1 mM]. Thus, precursors for LPA production are abundant. Indeed, incubation of plasma or serum at 37°C results in a time dependent increase in both LPC and LPA [Aoki et al., 2002]. However, despite the high concentrations of precursors, LPA levels are maintained at a low level. LPA production by plasma may be constrained in an inactive state by the presences of inhibitors [Sano et al., 2002]. This is consistent with a decreased rate of production of LPA in plasma as compared to serum [Aoki et al., 2002].

The source of phospholipids and lysophospholipids in plasma and sera that contribute to LPA production under physiological and pathophysiological conditions is not completely clear. Hepatocytes produce large amounts of lipids and lysophospholipids. LDL and HDL contain phospholipids, which can be converted to lysophospholipids by oxidation or by the action of sPLA2 or PS-PLA1 [Natarajan et al., 1995; Siess, 1999]. Indeed a number of responses to oxidized LDL or lysophospholipids such as LPC could potentially be mediated by conversion to LPA [Natarajan et al., 1995; Siess, 1999]. The still controversial suggestion that LPC-specific GPCRs exist [Kabarowski et al., 2001; Zhu et al., 2001; Bektas et al., 2003; Ludwig et al., 2003] suggests that LPC may mediate cellular signaling in addition to being a precursor for LPA.

A number of cell types produce lysophospholipids under physiological conditions. In blood cells, activation of platelets with thrombin or calcium ionophores results in the release of lysophospholipids [Aoki et al., 2002; Sano et al., 2002]. Erythrocytes can release small amounts of LPC. Both lecithin cholesterol acyltransferase

and platelet-activating factor acetylhydrolase (PAF-AH) have been implicated in production of LPC in plasma and sera. Studies by Aoki and colleagues using blood from patients deficient in LCAT and PAF-AH demonstrate that both LPC and LPA production is deficient in plasma from LCAT-deficient but not PAF-AH deficient patients [Aoki et al., 2002]. However, it is important to note that while basal LPC levels are slightly depressed in LCAT-deficient patients, they still are in the 100 μM range suggesting that plasma LPC originates from a source other than LCAT. Further the 100 μM of LPC present in plasma is far in excess of that required for the production of LPA. Patients with LCAT deficiency exhibit kidney dysfunction, anemia, and corneal opacification along with aberrations in plasma composition and amounts of cholesterol and other lipids [Gjone, 1982]. It is not clear whether LCAT-deficient patients exhibit decreased agonist induced LPA production in pathophysiological states and whether any of the consequences of the syndrome are due to LPA-deficiency. Indeed, these patients do not exhibit aberrations in processes proposed to be mediated by LPA such as wound healing or vessel development [Gjone, 1982].

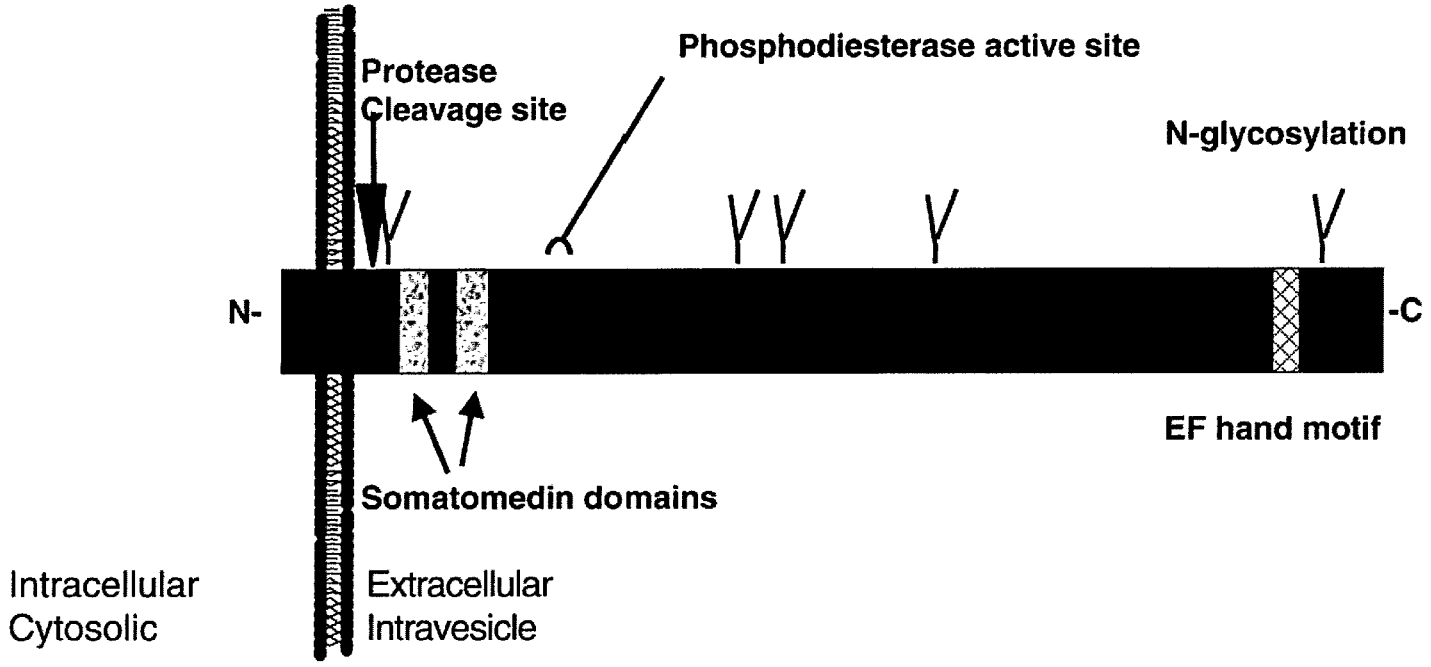
In an independent pathway, PA produced by the action of PLD on cellular membranes can be hydrolyzed by sPLA2 or PA-specific PLA1 (PA-PLA1) to produce LPA [Hiramatsu et al., 2003]. The magnitude of the contribution of this pathway in physiologic or pathophysiologic conditions remains unknown (Fig. 1). A fluorogenic PLA1/2 assay using a PC analogue has been developed [Feng et al., 2002], and a fluorogenic substrate for PA-specific PLA activity has also been synthesized [Xu et al., 2004]. These assays can be used to determine activity or screen for inhibitors.

LysoPLD/Autotaxin

Plasma and sera contain a lysophospholipid specific PLD (lysoPLD), which cleaves lysophospholipids to produce LPA [Xu et al., 1998; Aoki et al., 2002]. LysoPLD exhibits a preference for lysophospholipids containing unsaturated fatty acyl chains and may thus contribute to the preferential increases in the unsaturated fatty acyl chain containing LPA present in plasma and sera. LysoPLD activity is increased in women in the third trimester of pregnancy and in women at risk for preterm labor suggesting that levels of lysoPLD can vary under physiological and pathophysiological conditions [Tokumura et al., 2002a]. LysoPLD activity is also increased in rabbits fed a high cholesterol diet [Tokumura et al., 2002b]. As LPA also increases attachment of monocytes to vascular endothelial cells, and alters proliferation, and differentiation, of vascular smooth muscle cells and is present in atherosclerotic plaques, lysoPLD with subsequent LPA production may contribute to atherosclerosis [Tokumura et al., 1994; Siess et al., 1999].

The cloning of LysoPLD and its identification as autotaxin (ATX, nucleotide phosphodiesterase/pyrophosphatase, NPP2, phosphodiesterase-Ialpha) by the Aoki and Tokumura groups [Tokumura et al., 2002c; Umezu-Goto et al., 2002] opened a new era in our understanding of the metabolism and, significantly, the function of LPA. ATX had been previously cloned from melanoma cells and studied by the Liotta group and others as autocrine motility factor, an inducer of cell motility, angiogenesis, invasiveness, metastasis, and tumor aggressiveness [Murata et al., 1994; Nam et al., 2000, 2001; Yang et al., 2002b]. Indeed, in retrospect, the actions and mechanisms of signaling of ATX and LPA demonstrated remarkable concordance suggesting an overlap in mechanisms. ATX is a member of the nucleotide phosphodiesterase/pyrophosphatase family, but in contrast to other members of the family, it exhibits the novel ability to hydrolyze LPC and sphingosylphosphorylcholine (SPC) to produce LPA and sphingosine 1-phosphate (S1P). All of the demonstrated activities of ATX appear to be related to its ability to hydrolyze lysophospholipids rather than to its nucleotide phosphodiesterase/pyrophosphatase activity [Koh et al., 2003]. At least in model systems, the relative production of S1P by ATX can inhibit the migratory effects of LPA suggesting that the outcome of autotaxin action may reflect the relative production of LPA and S1P [Clair et al., 2003].

ATX, a 125 kDa type II transmembrane protein, has a short intracellular domain and a large extracellular domain with the catalytic site localized near the membrane in the extracellular domain and thus readily accessible to extracellular LPA and S1P as well as to inhibitors and drugs (Fig. 2). While the mechanism of catalysis of ATX is well characterized [Koh et al., 2003], its post-translational processing, regulation of expression and mechanism of



- 125 kDa type II membrane protein and released from membrane.
- 5'-nucleotide pyrophosphatase / phosphodiesterase family (NPP-2)
- Widely expressed
- Upregulated by bFGF, BMP-2, retinoic acid and Wnt-1signaling
- Stimulates tumor cell motility, metastasis and angiogenesis
- Metastatic capability relates to ATX levels in breast cancer
- Ectoenzyme Potential therapeutic target

Fig. 2. Characteristics of ATX/LysoPLD ATX is released from cells due to perimembrane cleavage. The mechanism and whether this happens intracellularly or at the cell surface is unknown as is the enzyme involved. The major action site of action of ATX is nevertheless extracellular.

release from cells are not well understood. Further, the relative activity of cell surface versus cleaved ATX is unknown. It is also not clear whether cleavage of ATX occurs in intracellular organelles or at the cell surface. Histochemical analysis suggests that that majority of ATX is localized in intracellular organelles rather than on the cell surface (not presented). However, as noted above, ATX and lysoPLD activity is found in bodily fluids and cell supernatants indicating that autotaxin is released from cells either following cleavage in organelles.

As assessed by SAGE analysis (http://cgap.nci.nih.gov/Sage/Viewer), levels of ATX are low in most cell lineages. Spinal cord has the highest level of expression of ATX suggesting a novel function in differentiated cells. Significant levels of ATX mRNA are also present in brain, breast, prostate, and hematopoietic cells. ATX can be upregulated by a number of cellular stimuli including growth factors such as bFGF and BMP2, retinoic acid and the WNT pathway [Bachner et al., 1998; Tice et al., 2002], however, the role of ATX and LPA in the function of these mediators is unknown. ATX can regulate proliferation of preadipocytes, and its production is increased during adipocyte differentiation and obesity implicating LPA production in obesity [Gesta et al., 2002; Ferry et al., 2003]. The mechanisms regulating ATX upregulation by these factors is unclear, however, MKK7, JNK, and Jun have been implicated in the process by several different approaches [Wolter et al., 2002; Black et al., 2004].

An analysis of ATX mRNA levels using a publicly available transcriptional profiling database (http://www.gnf.org/cancer/epican) indicates that levels of ATX mRNA are low in adult epithelial cells derived from a variety of tissues [Su et al., 2001]. As compared to the levels in normal epithelium, ATX mRNA levels are remarkably increased up to several thousand folds in kidney (renal cell carcinoma) tumors suggesting an important role in this tumor lineage (Fig. 3, Table I). Suppressed subtractive hybridization had previously demonstrated over expression of ATX in renal cell carcinoma [Stassar et al., 2001] compatible with these results. Renal cell carcinomas are amongst the most vascular tumors and contain high levels of angiogenic factors suggesting a potential role for LPA-mediated production of angiogenic factors such as VEGF, IL8, and IL6 in this disease [Takahashi et al., 1994]. This is compatible with our observations that LPA is a potent induced of angiogenic factors including VEGF, IL8, and IL6 and with the high levels of VEGF present in ovarian cancer ascites [Zebrowski et al., 1999; Hu et al., 2001; Schwartz et al., 2001; Fang et al., 2003]. According to the SAGE database, ATX mRNA levels are low to undetectable in normal kidney suggesting that the increased levels of autotaxin mRNA in renal cell carcinoma represent a novel acquisition of expression. ATX mRNA levels are more modestly but still markedly (100 fold) increased in a broad spectrum of cancers, including liver, gastric, ovary, lung, liver, prostate, and bowel (Fig. 3 and Table I). The modestly elevated levels in ovary and prostate may contribute to the autocrine LPA loops present in both of these tumors [Fang et al., 2002; Mills et al., 2002; Xie et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003]. It is important to note that within a given tumor type expression levels vary markedly with only modest or no increases in some tumors coupled with remarkable increases at least hundred fold in others. Transcriptional profiling data from Stanford [Schaner et al., 2003] and our own group indicates at least a 2 fold increase with a range of 1–12 in more that half of ovarian cancers as compared to normal ovarian epithelial cells. The discrepancy in relative increases between data sets likely arises from the low levels of ATX mRNA in normal tissues and need to use this as a comparator. Nevertheless, this confirms that ATX mRNA levels are increased in a number of cancer lineages.

We thus assessed ATX levels in a number of tumor cell lines using quantitative PCR. As indicated in Figure 4, ATX mRNA levels vary markedly among tumor cell lines [IOSE29 is used to represent a normal, non-transformed cell line, Auersperg et al., 1999]. However, compatible with the transcriptional profiling data, ATX levels were markedly elevated in a subset of tumor cell lines, particularly glioma and renal cell carcinomas. Even in these two cell lineages, some tumors show limited increases in ATX levels. More modest increases are observed in ovarian cancer cell lines with only limited changes in a subset of breast lines.

If the marked increase in mRNA levels of ATX in glioma and renal cell carcinoma (Figs. 3 and 4) results in release of active enzyme and diffusion into the plasma, ATX levels and ATX activity

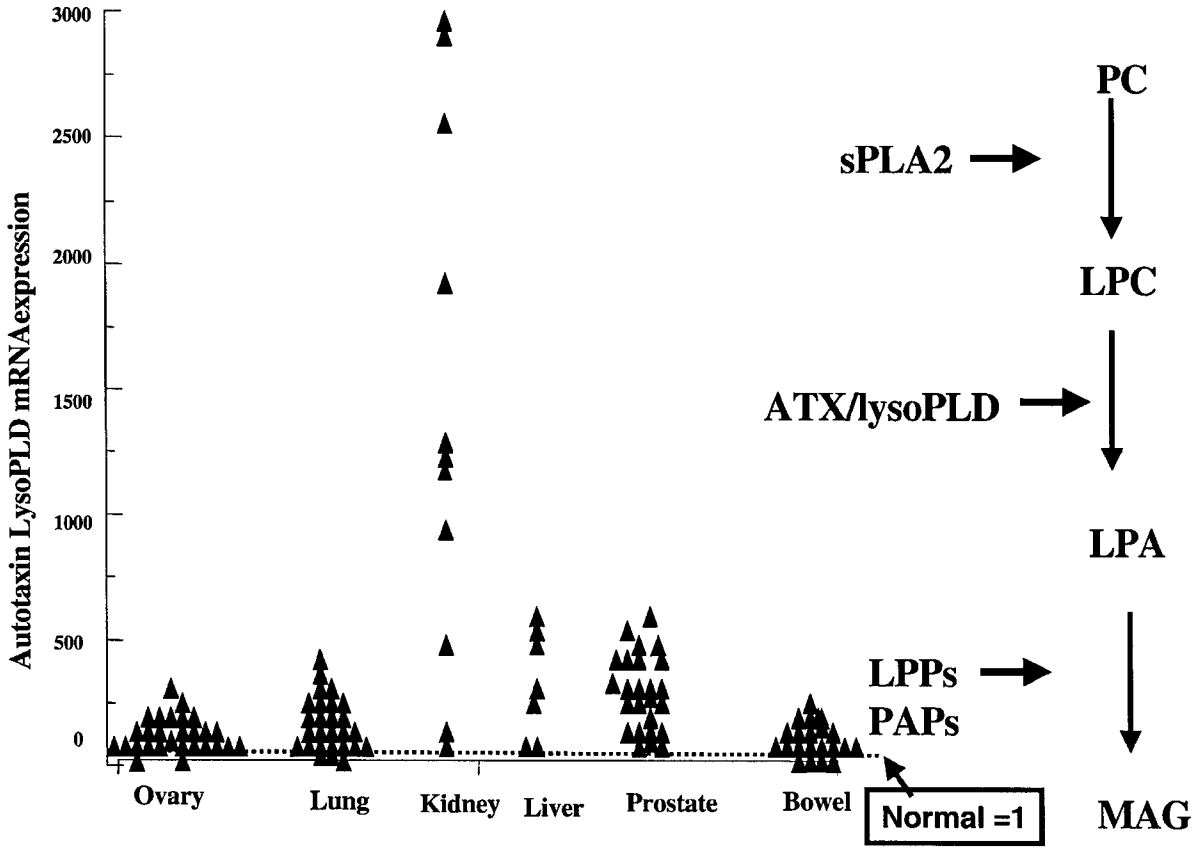


Fig. 3. ATX/LysoPLD mRNA levels are increased in cancer using a publicly available database of Affymetrix U95 arrays (http://www.gnf.org/cancer/epican 74), autotaxin mRNA levels are shown to be markedly aberrant in a number of tumor types. Data is expressed for each tumor relative to control values based on 36 normal adult epithelial tissues. As indicated in the figure, a number of tumors from each cell lineage express elevated levels of autotaxin mRNA.

could be markedly increased in a number of tumor types. To rapidly quantify the lysoPLD activity of ATX in patient samples, a novel fluorogenic assay was developed using the principles developed earlier for PLA activity measurement [Feng et al., 2002]. We thus evaluated ATX enzyme activity assay based on a quenched dye approach detecting the release of fluorescent ethanolamine from its quencher on a synthetic LPE [Drees et al., 2003; see Fig. 5 legend]. OVCAR3 express modest levels of ATX (Fig. 4) and also produces low levels of LPA in culture [Eder et al., 2000], compatible with the basal level of ATX activity. As indicated in Figure 6, cell supernatants from OVCAR3 cells induced a time dependent increase in fluorescence due to unquenching of the fluorescent dye. Further, fetal bovine serum, which contains high levels of ATX and supernatants from lysoPLD/ATX transfected OVCAR3 cells demonstrated increased lysoPLD activity (Fig. 6). This assay was used to assess the levels of ATX activity in plasma and sera samples from controls and patients. As indicated in Figure 7, levels of ATX activity in ovarian cancer ascites were markedly elevated as compared to plasma and sera. There was a statistically significant increase in ATX/lysoPLD activity in the plasma of patients at diagnosis with ovarian cancer and glioma but not in patients with breast cancer similar to the ATX mRNA levels in tumors and cell lines from these cancers (Fig. 7). Similar results were obtained for serum, which had modestly elevated activity as compared to plasma suggesting that ATX is activated (derepressed) or released from blood cells during the coagulation process. However, the increase in ATX activity in serum and plasma from cancer patients were modest as compared to normal controls and exhibited significant overlap

TABLE I. Analysis of Gene Expression Profiles of *LPP* Genes in Different Tumor Samples From Published Microarray Hybridization Data

	Type of cancer									
	Ovarian	Breast	Colon	Prostate	Kidney	Pancreas	Bladder	Liver	Gastric	Lung
Enzyme										
LPP-1	0.2 + 0.1	0.4 + 0.1	0.3 + 0.1	6.0 + 2.15	2.6 + 1.3	0.7 + 0.2	1.6 + 0.8	1.0 + 0.3	0.4 + 0.4	0.5 + 0.3
LPP-2	1.1 + 1.2	2.8 + 1.5	3.1 + 1.0	1.0 + 1.0	0.4 + 0.8	3.7 + 1.3	2.0 + 1.1	0.6 + 1.6	2.1 + 1.8	2.2 + 1.0
LPP-3	1.0 + 1.1	0.6 + 0.5	0.5 + 0.4	2.9 + 1.2	4.7 + 2.6	0.9 + 0.4	1.2 + 0.8	1.8 + 2.3	0.2 + 0.4	1.1 + 0.4
ATX	34.3 + 32	27.7 + 27	20.7 + 17	73.8 + 46	689 + 493	61.4 + 37	58.5 + 59	121 + 95	24.6 + 35	56.6 + 43.5

Affymetrix U95 array hybridization data are publicly available on 10 different primary human cancers and normal epithelial samples (http://www.gnf.org/cancer/epican 74). The control samples consisted of 36 normal adult epithelial tissues. To allow comparison across multiple tumor types, the mRNA expression data of each gene was normalized to the expression detected in normal epithelial cells. The data is expressed as average of expression ± standard deviation relative to normal epithelial cells. The sample numbers varied between from 6 to 28 for different tumor types.

with ATX levels in normal individuals (Fig. 7) suggesting that an analysis of ATX activity, at least by this approach, in serum or plasma would not provide clinically relevant data. This is in concordance with an earlier report on ovarian cancer sera that failed to detect clinically relevant increases in lysoPLD activity [Tokumura et al., 2002d].

Both LPA and ATX, through LPA production, have been implicated in the activation, proliferation and survival of several cancer cell lineages, but have not been assessed in the pathophysiology of renal cell carcinoma. As indicated above, the ability of ATX and LPA to induce the production of angiogenic factors combined with the high level of these factors produced by renal cell carcinoma cells suggested that ATX and LPA may be critical players in renal cell carcinoma. Given the high level of ATX mRNA in renal cell carcinomas and in the UM-RC-7 renal cell carcinoma cell line (Fig. 4), we explored the effects of decreasing ATX levels with siRNA on cellular signaling and on cell cycle progression and apoptosis in UM-RC-7 cells. As indicated in Figure 8, siRNA specific to autotaxin markedly downregulates ATX expression, an effect that persists for at least 72 h. Compatible with decreased endogenous LPA production, activated MAPK and AKT levels decreased, albeit with greater decreases at later time points (Fig. 8) (total AKT and total MAPK serve as loading controls indicating that equal amounts of proteins were assessed). The delayed decrease in cell signaling as compared to ATX levels may reflect persistence of extracellular ATX or of LPA in supernatants. Nevertheless, siRNA effectively downregulates ATX expression in UM-RC-7 cells and down regulation of ATX is associated with a marked decrease in cell signaling.

We thus explored the effects of down regulation of ATX on cell cycle progression and apoptosis as indicated by cells with a hypodiploid DNA content. In UM-RC-7 cells in both the presence and absence of sera, down regulation of ATX, resulted in an arrest of cells in S phase with an accumulation of apoptotic cells as indicated by an increase in hypodiploid (sub G0/G1) cells (Fig. 9). Similar increases in apoptosis and accumulation of cells in S phase were noted in both glioma and breast cancer cells (not presented). The marked accumulation of cells in S phase was unexpected and suggests activation of the intra-S cell cycle checkpoint, an enigmatic

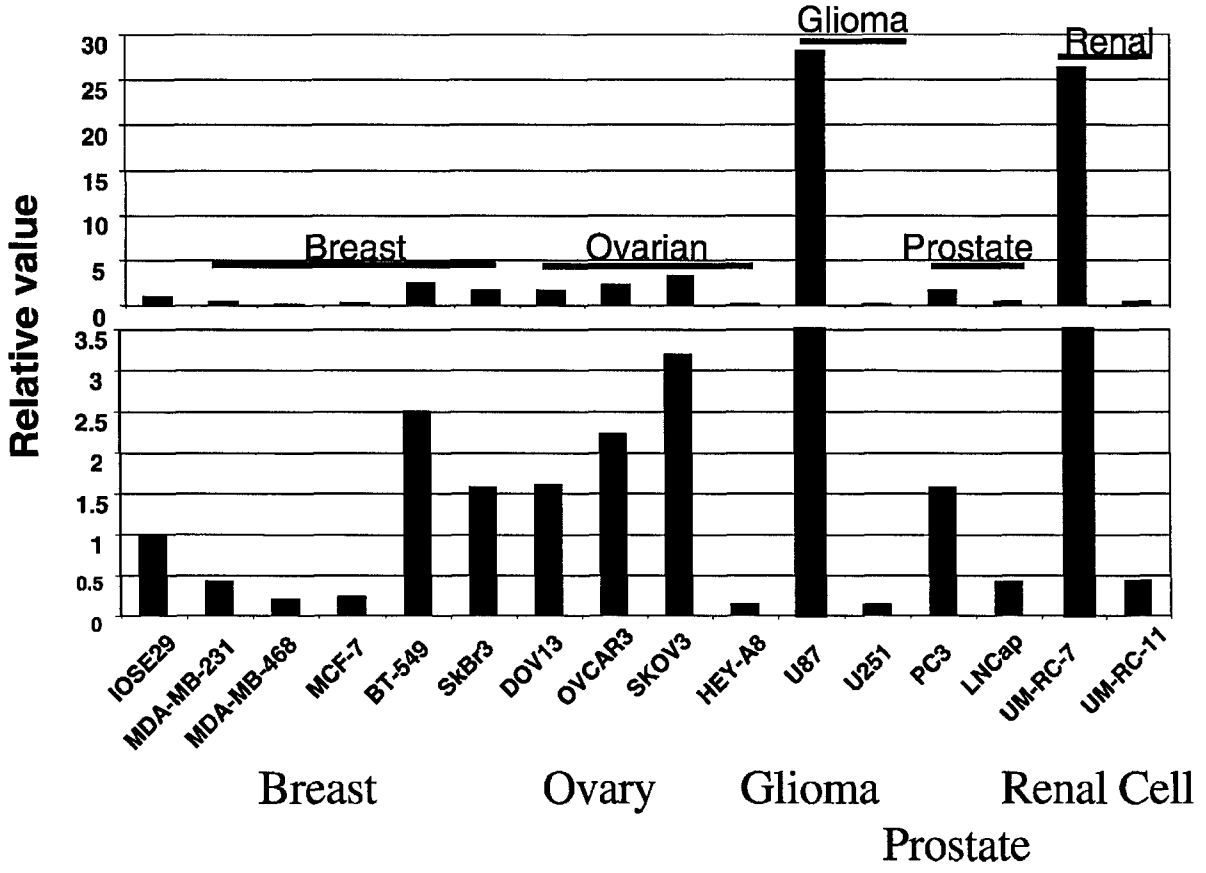


Fig. 4. LysoPLD/ATX RNA levels in tumor cell lines Quantitative PCR was performed on a series of breast, ovarian, glioma, prostate and renal cancer cell lines. Data is normalized to IOSE 29, a SV40 T antigen semi-immortalized cell line that is set to 1. As indicated in the figure, the levels of ATX/lysoPLD mRNA are variable between cell lines including within specific tumor types. U87, a glioma cell line, and UM-RC-7, a renal cell line, express markedly elevated levels of ATX/lysoPLD RNA levels.

check point previously implicated in repair of DNA damage. We have previously overexpressed LPA phosphatases (LPPs) in ovarian cancer cells [see below Tanyi et al., 2003a,b], which increase LPA hydrolysis and should thus have similar effects to autotaxin siRNA that decreases LPA production. Indeed, overexpression of LPPs resulted in an accumulation, albeit more modest that ATX siRNA, in cells in S phase as well as increased apoptosis. The mechanism leading to an increased in cells in S phase is unknown and the topic of ongoing studies.

Taken together, ATX mRNA levels are markedly elevated in a number of cancer cell lineages. Although ATX activity is increased in the plasma and serum from patients with particular types of cancer, the increases are modest and unlikely to be clinically or diagnostically relevant. However, increased autotaxin activity at the tumor cell interstitial fluid interface is likely to result in increased production of LPA. Indeed, in ovarian cancer patients, genes that are co-regulated with ATX comprise an LPA-dependent transcriptome (not presented), potentially providing a method to identify LPA-dependent tumors likely to be responsive to manipulation of ATX activity or LPA expression. In tumor cell lines over expressing ATX, silencing of ATX with siRNA to ATX results in decreased transmembrane signaling, S phase arrest, and accumulation of apoptotic cells. This combined with previous data implicating ATX in tumor proliferation, motility, aggressiveness, and metastases validates autotaxin and by implication LPA production and action as a target for tumor therapy. As ATX functions as an extracellular enzyme, it is highly accessible for inhibition by antibodies, peptides, pseudosubstrates, or small molecule drugs.

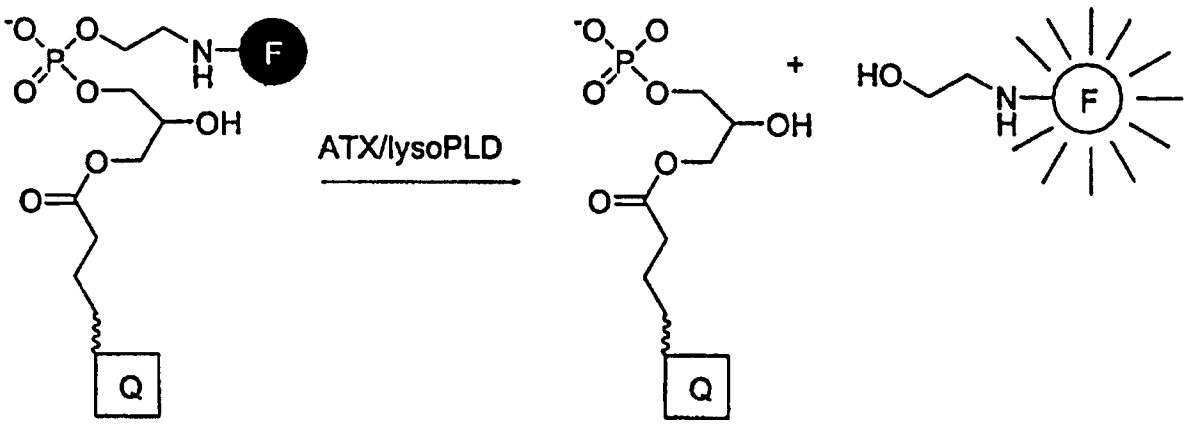
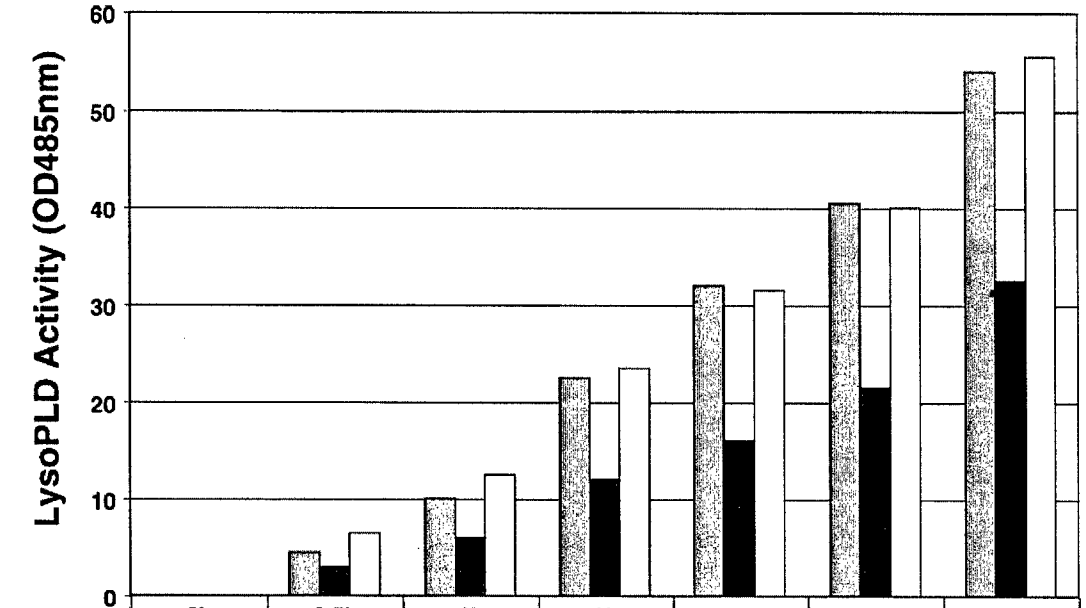


Fig. 5. Quenched fluorescence lysoPLD enzyme assay. A quenched cleavable substrate assay similar that developed for PLA2 [Feng et al., 2002] was established for LysoPLD. A doubly labeled substrate analog based on LPE was synthesized containing fluor and quencher moieties. The close intramolecular proximity of the quencher and fluorescent groups results in effective quenching of fluorescence by energy transfer in the substrate. Upon digestion by lysoPLD, the ethanolamine group containing the fluor is released, energy transfer is interrupted, and fluorescence increases as a function of the extent of lipase activity.

LPA Degradation

LPA can be removed by cleavage of the acyl chain by phospholipases, reacylation by LPAAT or endophilin, or by hydrolysis of the phosphate by lysophosphatidic acid phosphatases [LPP-1, -2, -3 also know and PAPs or PA phosphatases, Brindley et al., 2002; Mills et al., 2002; Tanyi et al., 2003a,b; see studies in this review]. LPPs are hexahelical transmembrane lysolipid phosphatases with an extracellular catalytic site, suggesting a role in hydrolysis of extracellular LPA. Although different LPPs demonstrate modest selectivity for lysophospholipid isoforms, each of the LPP isoforms can hydrolyze LPA, S1P or ceramide 1-phosphate and likely other lysophospholipids. They exhibit little activity however toward PA or other diacyl chain lipids. Over 95% of hydrolysis of LPA by cancer cells appears to be due a membrane associated LPP activity suggesting that LPPs are the major method for clearance of LPA in vivo [Imai et al., 2000]. As indicated in Table I (relative levels) and Figure 10 (absolute levels), LPP mRNA levels and in particular LPP-1 mRNA levels demonstrate marked variability in different tumor lineages. For example, LPP levels are elevated in prostate and kidney cancers whereas they are markedly decreased in ovary, lung, and breast cancer. Intriguingly, prostate and renal cell carcinomas, which have high levels of autotaxin mRNA (Table I, Fig. 3) also exhibit elevated levels of LPP-1 mRNA. Thus there is likely to be an increased production and hydrolysis of LPA in these tumor types with potentially increased information flow through the pathway. What the net effect of overexpression of both ATX and LPP-1 is on LPA production and degradation is remains to be assessed. LPP-1 levels are markedly decreased in ovarian cancer (Table I, Fig. 10), potentially contributing to the elevated levels of LPA present in ascites. We have explored the role of LPPs in ovarian cancer by increasing expression of LPP-1 and determining effects on cellular functions [Tanyi et al., 2003a,b]. In most ovarian cancer cells, overexpression of LPPs is incompatible with cell proliferation and survival. Overexpression of LPP-1 or LPP-3 in SKOV3 cells which have high levels of ATX (Fig. 4) and produce LPA spontaneously [Eder et al., 2000] is tolerated by the cells. Stable LPP overexpression decreases LPA levels, increases LPA hydrolyses, inhibits cell cycle progression resulting in an S phase arrest and accumulation of apoptotic cells and decreases growth subcutaneously and intraperitoneally [Tanyi et al., 2003a,b]. In a typical experiment (Fig. 11), transient overexpression of LPPs markedly decreases colony forming cell activity. Strikingly, this effect is reversed by addition of XY-13 (Fig. 11) and XY-14, two enantiomeric phosphonate analogues of PA [Xu and Prestwich, 2002], that act as potent inhibitors of the



	0h	0.5h	1h	2h	3h	4h	6h
LysoPLD	0	4.5	10	22.5	32	40.5	54
mock	0	3	6	12	16	21.5	32.5
FBS	0	6.5	12.5	23.5	31.5	40	55.5

The culture media from ATX / LysoPLD or control vector transfected OVCAR3, and FBS were subjected to LysoPLD assay. They showed time-dependent increase in activity and an increase in transfected cells.

Fig. 6. Validation of the lysoPLD assay. The quenched cleavable lysoPLD assay described in Figure 1, was validated by incubation with supernatants from lysoPLD and mock transfected OVCAR3 cells (OVCAR3 cells have modest lysoPLD mRNA levels) as well as with fetal bovine serum, which has high levels of lysoPLD [Umezu-Goto et al., 2002]. As indicated in the figure, following background subtraction there is a linear time dependent increase in lysoPLD substrate cleavage.

LPPs [Smyth et al., 2003]. The effect is also reversed by OMPT an LPP-resistant LPA analog [Hasegawa et al., 2003] that shows enantioselective activation of LPA3 [Qian et al., 2003] confirming that the effect of the LPPs is through degradation of LPA (Fig. 11). In support of hydrolysis of LPA as a mechanism of action, overexpression of LPPs in SKOV3 cells resulted in a marked decrease in the proliferation and survival of non-transfected SKOV3 cells, a bystander effect. This extracellular mechanism of action and strong bystander effect is optimistic for potential gene therapy approaches to ovarian cancer, a process being explored by our group.

The combined studies with autotaxin siRNA and overexpression of LPPs provide strong evidence validating the LPA pathway as a target for therapy in cancer. This suggests that drugs targeting autotaxin, which is relatively specific for LPA production (SPC levels are low extracellularly compared to LPC), or specific LPA receptors or their downstream signaling pathways may have efficacy in the therapy of cancer [Tokumura et al., 2002c; Umezu-Goto et al., 2002; Clair et al., 2003]. As indicated above, LPA and autotaxin have pleiomorphic effects in multiple cell lineages, suggesting that inhibition of the LPA production or action may be toxic. However, knockouts of LPA1 and -2 and indeed combined LPA1 and -2 knockout mice are viable suggesting either that these pathways are not required for normal cellular physiology or that LPA3 and -4 exhibit redundant activities [Yang et al., 2002a]. These questions will be answered through the development

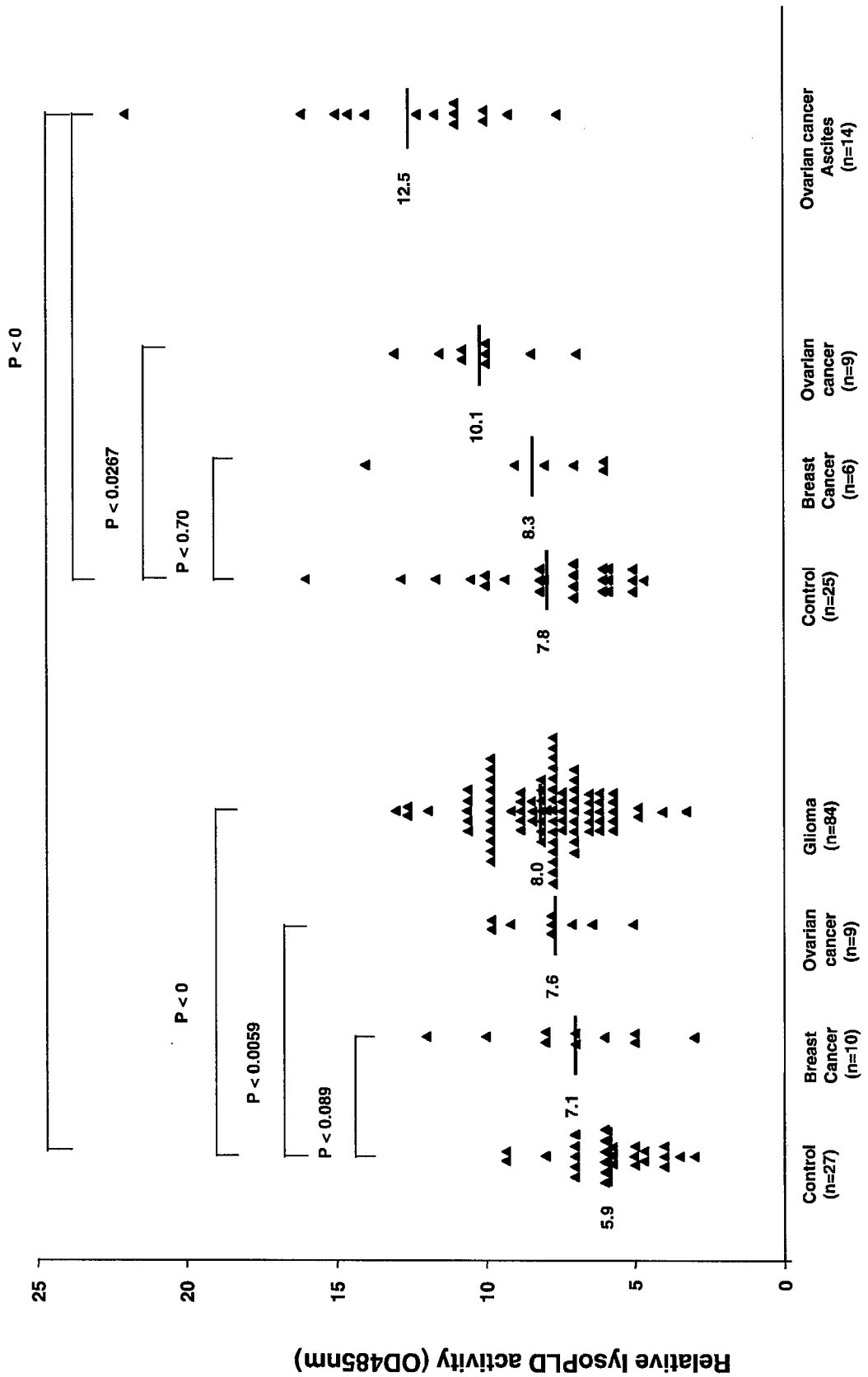


Fig. 7. LysoPLD activity in patient samples using the validated lysoPLD assay. lysoPLD enzyme activity was measured in serum and plasma of patients with glioma, ovarian cancer or breast cancer. Serum and plasma activity was assessed for breast and ovary and ascites levels for ovarian cancer. Mean levels are indicated by a bar for each set. Statistically different results (Student's *t*-test) are indicated in the figure.

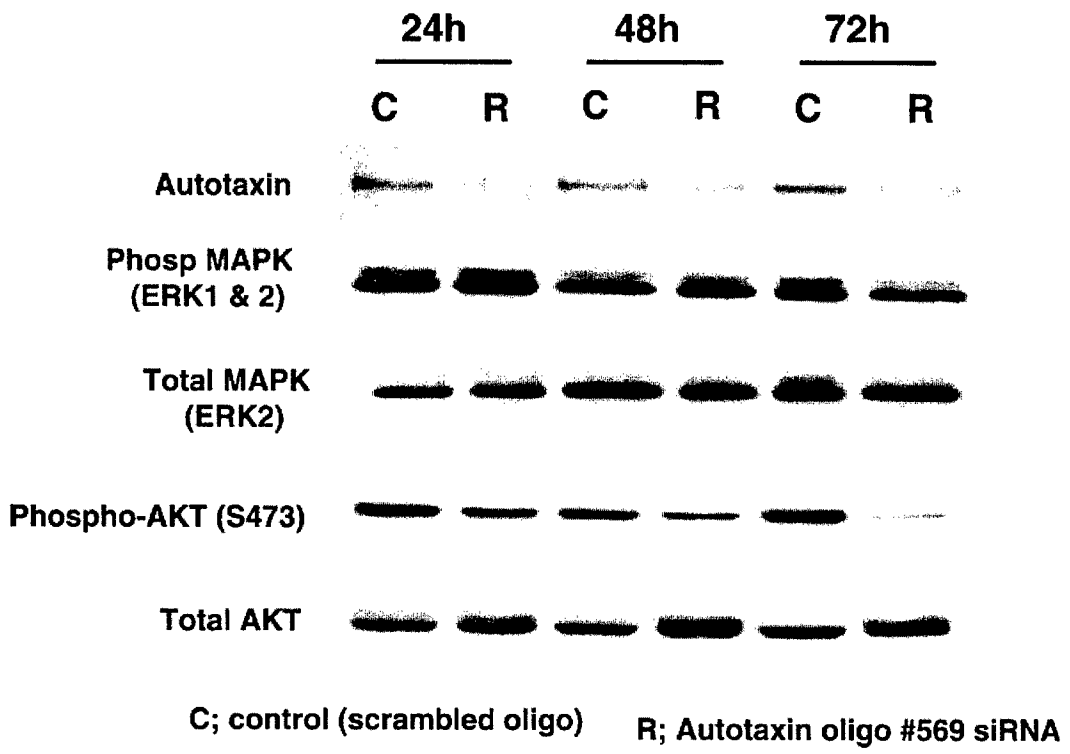


Fig. 8. RNAi to ATX /lysoPLD decreases ATX levels and decreases signaling to AKT and MAPK RNAi specific to autotaxin was introduced into UM-RC-7 renal cell carcinoma cells that overexpress autotaxin (Fig. 4). The RNAi induced a prolonged and significant downregulation of autotaxin protein levels in the cell line. This decrease was associated with decreased phosphorylation of both erk1 and erk2 as well as AKT. There was no decrease in total MAPK or AKT demonstrating equal loading and lack of non-specific effects.

and assessment of small molecule inhibitors and agonists of specific LPA receptors, a process which we have recently reviewed [Feng et al., 2003].

LPA in Ovarian Cancer

Ovarian cancer has been extensively explored as a model for aberrations in LPA production, metabolism and function [Xu et al., 1995, 1998, 2003; Shen et al., 1998, 2001; Westermann et al., 1998; Eder et al., 2000; Xiao et al., 2000, 2001; Hu et al., 2001; Schwartz et al., 2001; Baker et al., 2002; Fang et al., 2002, 2003; Mills et al., 2002; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Sengupta et al., 2003]. Although it is clear that LPA plays a major role in the pathophysiology of ovarian cancer, data is rapidly accumulating [see above Mills and Moolenaar, 2003], implicating LPA in the pathophysiology of multiple different cancers. Nevertheless, due to the wealth of data in ovarian cancer, the remainder of this review will concentrate on the unique aspects of ovarian cancer and what is known about LPA production, metabolism, and action in ovarian cancer.

LPA levels. It is difficult to determine the levels of LPA and indeed any growth factor in the interstitial fluid of tumors and thus we have been limited in our ability to assess whether cancer cells, in vivo, are stimulated by particular growth factors. The ascitic fluid that accumulates in ovarian cancer patients provides access to the distinctive ovarian cancer microenvironment. The growth factor composition of ascitic fluid reflects growth factor production, degradation and clearance from the local environment and also reflects the growth factor environment of the tumor cell. We have demonstrated that malignant ascites from ovarian cancer patients contain high levels of growth factor activity both in vitro and in vivo [Fang et al., 2002; Mills et al., 2002]. At least a portion of the growth factor activity in ascites can be attributed to the presence of high concentrations of LPA and LPA like molecules present

72h	0% FBS		10% FBS	
	control	siRNA	control	siRNA
Sub G0 / G1	4.27%	9.73%	1.21%	3.92%
G0 / G1	88.34%	58.99%	90.10%	41.95%
S	4.67%	27.10%	5.57%	54.83%
G2 / M	6.98%	13.91%	4.33%	3.22%

Fig. 9. RNAi to ATX induces a S phase arrest and apoptosis. RNAi specific to ATX was introduced into UM-RC-7 renal cell carcinoma cells that overexpress ATX. Seventy-two hours later adherent and floating cells were collected and analyzed for cell cycle and apoptosis (hypodiploid peak) using flow cytometry of propidium iodide labeled cells. RNAi induced a marked accumulation of cells in S phase as well as hypodiploid cells. Similar results were obtained at 48 h.

in ascites (1–80 μM) [Westermann et al., 1998; Eder et al., 2000; Xiao et al., 2001; Fang et al., 2002; Mills et al., 2002; Mills and Moolenaar, 2003; Xu et al., 2003]. Ovarian cancer ascites not only contains consistently elevated levels of LPA, but the LPA present contains a number of unusual forms, which may have novel mechanisms of production or action [Xiao et al., 2001].

Since LPA levels are elevated in ascites of ovarian cancer patients, if LPA migrates to the periphery, there is a potential that LPA levels could be elevated in the plasma of patients providing an early diagnostic marker or marker of disease behavior. Indeed, LPA levels are consistently higher in ascites samples than matched plasma samples suggesting that LPA produced in the peritoneal cavity migrates into the plasma [Eder et al., 2000]. In a preliminary analysis with a limited number of patients and controls, LPA levels were elevated in more than 80% of stage I ovarian cancer patients with a false positive rate of 5% [Xu et al., 1998, 2003]. LPA levels are not elevated in blood samples from patients with most other cancers, but are increased in patients with myeloma, endometrial cancer, cervical cancer, and renal dialysis [Sasagawa et al., 1998, 1999], all of which can be distinguished from ovarian cancer by medical assessment. Following the original publication, a number of reports have supported or refuted the potential utility of LPA levels in early diagnosis and prognosis of ovarian cancer [Xiao et al., 2000; Shen et al., 2001; Baker et al., 2002; Yoon et al., 2003; Xu et al., 2003]. As plasma contains enzymes required for the production and metabolism of LPA [Xu et al., 1998; Aoki et al., 2002], differences in the results from the groups likely arise from challenges in the collection and handling of plasma to prevent post collection production, metabolism or loss of LPA. An additional complication arises in the analysis of LPA, which requires purification, concentration and multiple handling steps, which could result in artifactual changes in LPA levels. Current studies have focused on total LPA levels and levels of LPA isoforms. It

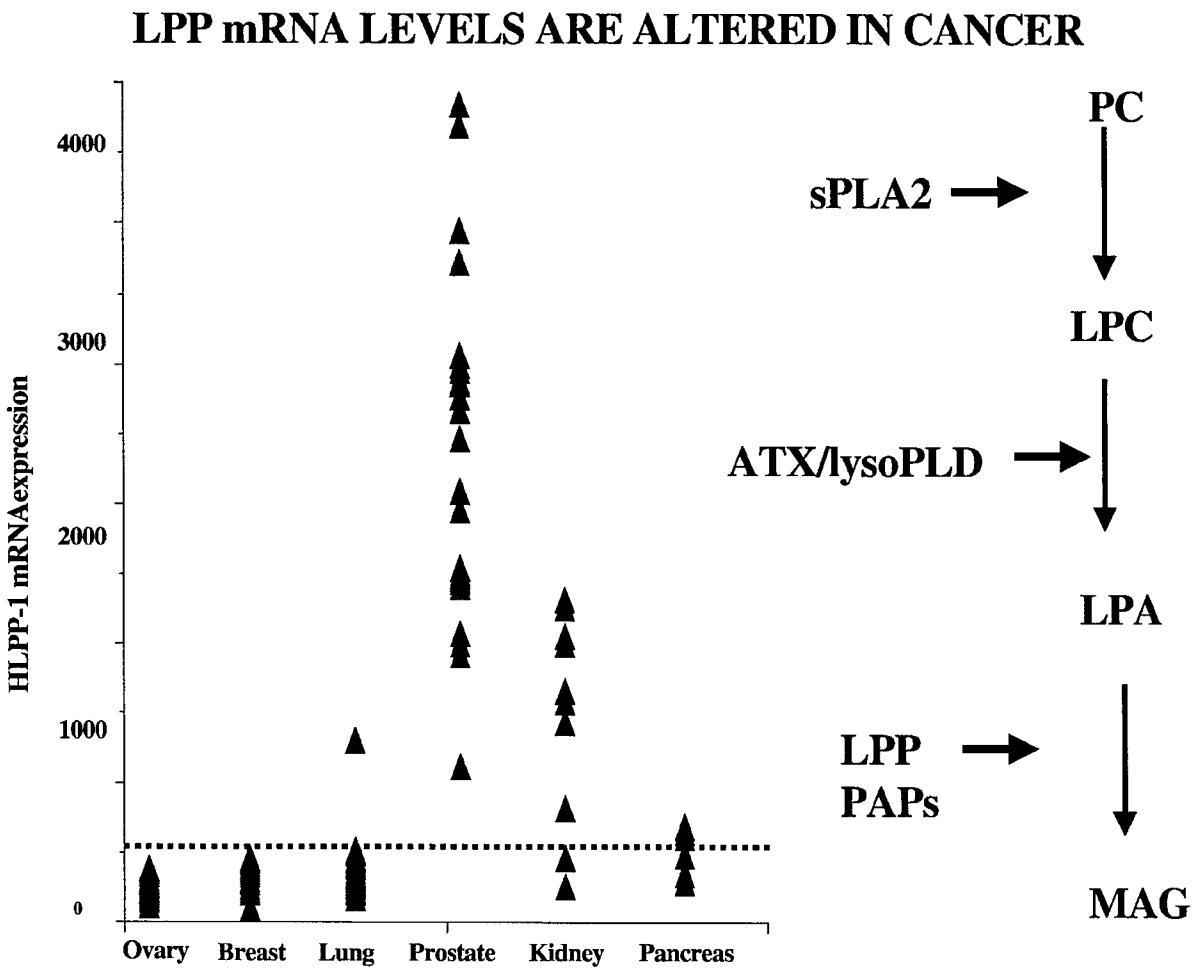


Fig. 10. LPP-1 mRNA levels are altered in cancer using a publicly available database of Affymetrix U95 arrays (http://www.gnf.org/cancer/epican), LPP-1 mRNA levels are shown to be markedly aberrant in a number of tumor types. Data is expressed for each tumor relative to control values based on 36 normal adult epithelial tissues. As indicated in the figure, LPP-1 mRNA levels are elevated in a number of tumor types, and decreased in others.

may, however, be necessary to assess the composition of particular isoforms of LPA or other lysophospholipids and related lipids precursors and breakdown products as part of a lipid profile to develop an algorithm able to detect clinically relevant changes in lipid levels or composition. Such approaches using global analysis of small molecules by mass spectroscopy has been designated metabolomics and a release of this technology as a clinically available "home brew" screening test for ovarian cancer is being proposed for early 2004. A reanalysis of data and follow up data from the original study describing the use of pattern recognition of small molecules by SELDI-TOF mass spectroscopy as a potential approach to detect ovarian cancer [Petricoin et al., 2002], suggested that a number of different molecules in the range of 200–800 Da had discriminatory power for the detection of ovarian cancer. A recent follow up study, indicates that the majority of these molecules are carried in the blood stream bound to albumin and other carrier proteins [Mehta et al., 2004]. LPA associates with albumin as well as with other carrier proteins such as gelsolin [Goetzl et al., 2000] suggesting that it may be in the carrier protein compartment. Combined with the observation that fatty acyl chains (200–300 Da), monoacylglycerols (300–400 Da), lysophospholipids (400–600 Da), and phospholipids (700–900 Da) fall into this range, it is possible that a

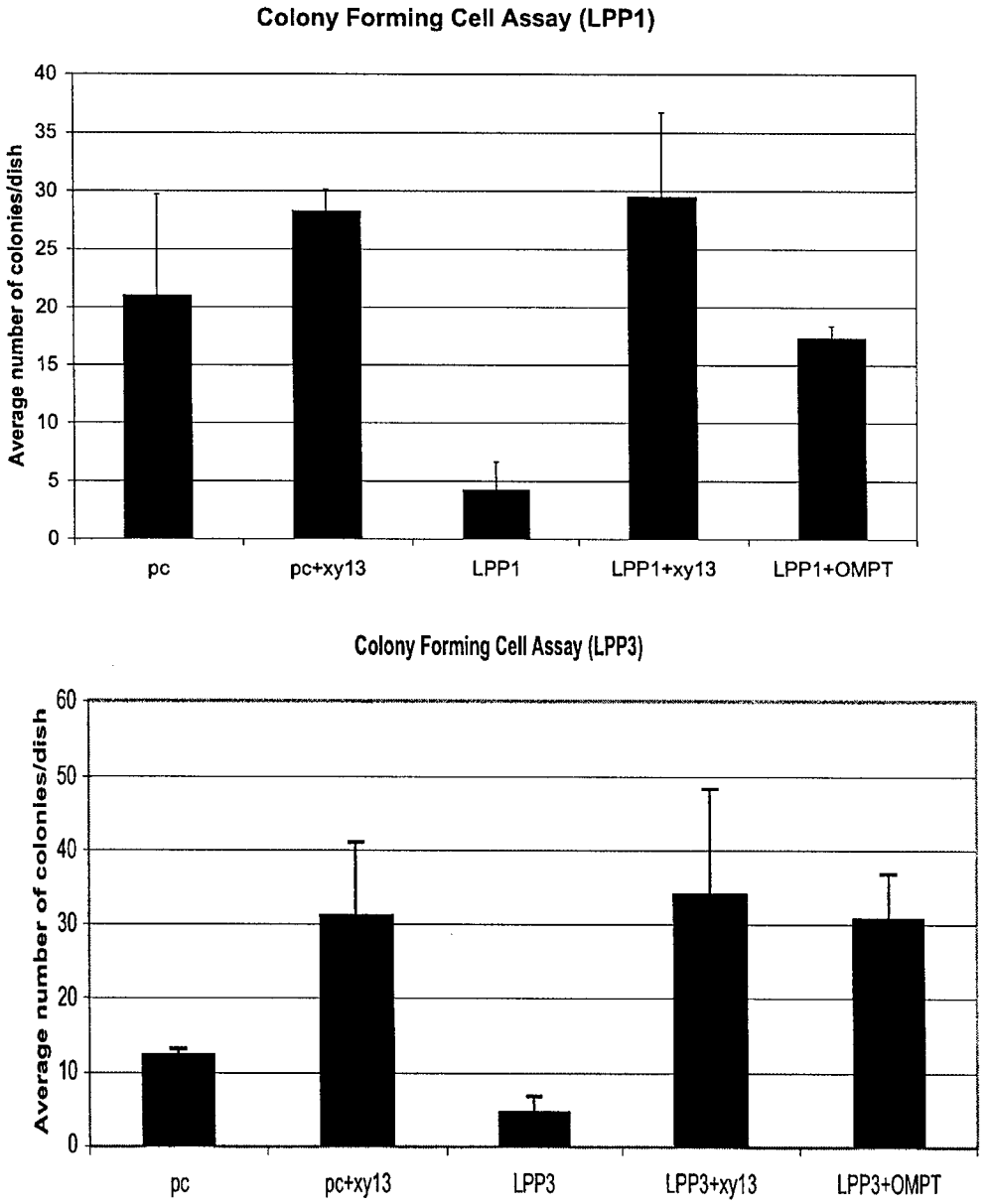


Fig. 11. LPP-1 and LPP-3 decrease colony forming cell activity of ovarian cancer cells through degradation of LPA. As indicated in Figure 10, LPP-1 levels are decreased in ovarian cancer (85). Transient transfection with LPP-1 or LPP-3 induces a marked decrease in colony forming cell activity in SKOV3 ovarian cancer cells, which express high levels of LysoPLD and produce LPA constitutively. Transfected cells were incubated with XY13 that inhibits LPPs and with OMPT, a LPP resistant LPA analog. Both XY-13 and OMPT reversed the effects of LPP on colony forming cell activity demonstrating that the effects of LPP-1 and LPP-3 were due to degradation of LPA.

global analysis of lipid patterns could prove diagnostic for particular cancers. We have demonstrated that model lipids bind with high affinity to the matrices used in SELDI, are excited by the laser and are easily visualized by SELDI-TOF. A prospective clinical trial to evaluate the utility of assessment of patterns of lipids in plasma measured by ESI (metabolomics), SELDI- or MALDI-TOF or other approaches as an ovarian cancer marker is clearly warranted.

Until recently the mechanisms resulting in high levels of LPA present in ovarian cancer ascites remained elusive. However, as indicated in Figure 12, a number of enzymes producing LPA including sPLA2 [Ben-Shlomo et al., 1997]

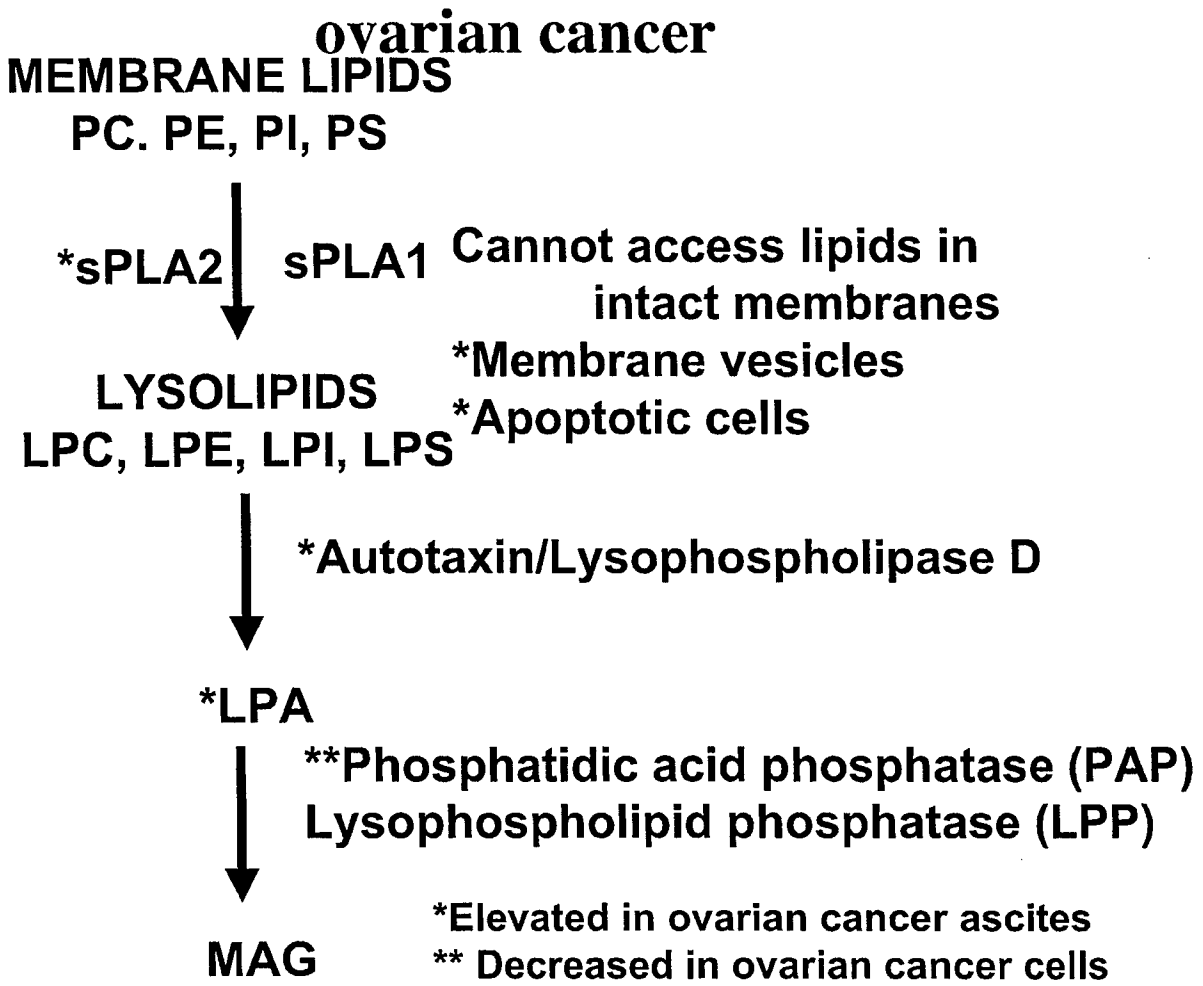


Fig. 12. Aberrations in lipid metabolism in ovarian cancer points of aberrations in lipid metabolism in ovarian cancer are indicated based on the major pathway of extracellular LPA production.

and autotaxin are increased in ovarian cancer patients (Fig. 3), and as noted above, LPP isoforms are decreased (Fig. 10) [Tanyi et al., 2003a,b]. Like many cancers, the number of apoptotic cells in ovarian cancers is high and membranes are accessible to the action of sPLA2 potentially resulting in increased production of substrates for ATX [Fourcade et al., 1995]. Ascites also contains markedly elevated levels of vesicles that are also accessible to sPLA2 [Ginestra et al., 1999; Andre, 2002]. Furthermore, ovarian cancer cells both constitutively and inducibly produce LPA [Shen et al., 1998; Eder et al., 2000; Sengupta et al., 2003]. As patients may have kilograms of tumor present when diagnosed, even a modest increment in production or decreased metabolism of LPA by ovarian cancer cells could result in marked accumulation of LPA in ascites. Indeed, ascites itself can contain up to 10^9 tumor cells per milliliter, which could result in release of high levels of LPA. Thus, there is a suite of aberrations in ovarian cancer that potentially contributes to the accumulation of LPA at the tumor interface and in ascites. Taken together, the data suggest that the increased levels of LPA in ovarian cancer ascites reflects both increased production and decreased hydrolysis.

LPA is not produced at significant levels by normal ovarian epithelial cells, whereas ovarian cancer cells both constitutively and inducibly produce elevated levels of LPA in response to phorbol esters, laminin and LPA itself [Shen et al., 1998; Eder et al., 2000; Sengupta et al., 2003], suggesting that ovarian cancer cells which present at great numbers in the peritoneal cavity could contribute to the elevated levels. The ability of ovarian cancer cells to

produce and respond to LPA suggests that ovarian cancer cells are likely regulated by autocrine LPA loops. A recent report confirms the presence of an autocrine LPA loop in prostate cancer [Xie et al., 2002] suggesting that LPA autocrine loops may contribute to the pathophysiology of multiple cancers.

In view of the higher levels of LPA in ascites from ovarian cancer patients and the ability of LPA to activate the pathways mediating LPA production, we assessed basal and LPA-induced LPA production by ovarian cancer cells. We found that ovarian cancer cells, in contrast to normal ovarian epithelial cells or breast cancer cells, produce LPA either constitutively or in response to LPA [Eder et al., 2000]. Both constitutive and LPA-induced LPA production exhibited PLD-dependent and -independent components. LPA has been demonstrated to activate PLD in a number of systems [van der Bend et al., 1992; Kam and Exton, 2004]. PLD can alter membrane composition and in particular induce production of membrane vesicles [Morgan et al., 1997; Kinkaid et al., 1998]. Indeed, ascites contains high levels of vesicles potentially due to PLD activation [Ginestra et al., 1999; Andre, 2002]. Constitutive LPA production was primarily dependent on group IB (pancreatic) sPLA2 and on cPLA2 and/or iPLA2, whereas LPA-induced LPA production was dependent on both group IB (pancreatic) and group IIA (synovial) sPLA2, but not cPLA2 or iPLA2 suggesting that LPA induces extracellular LPA production [Eder et al., 2000]. LPA is a potent activator of increases in cytosolic calcium and of MAPKs in ovarian cancer cells and both increases in cytosolic calcium and MAPK activity activate cPLA2 potentially contributing to the increases in LPA production. In contrast to LPA-induced LPA production, PMA-induced LPA production is dependent on cPLA2 or iPLA2 indicating effects on intracellular LPA production [Shen et al., 1998]. Laminin-induced LPA production by ovarian cancer cells is dependent on beta1 integrins and iPLA2 similar to PMA-induced LPA production [Sengupta et al., 2003]. Nucleotide agonists acting at the P2Y4 purinergic receptors on ovarian cancer cells also induce LPA production. Nucleotide induced LPA production is associated with an increase in PLD activity and PLD augments LPA production suggestion that it could be a major contributor to LPA production by ovarian cancers [Luquain et al., 2003b]. Ovarian cancer cells secrete PLA2. Indeed the increased concentration of vesicles in ascites that are accessible to sPLA could contribute to LPA production either by conversion of PA to LPA or through the production of LPC and other autotaxin substrates.

Aberrations in LPA Function in Ovarian Cancer

As noted above, the production of LPA is aberrant in ovarian cancer cells due to changes in the levels and activity of multiple different enzymes resulting in the accumulation of high concentrations of LPA in ascites fluid and likely in the interstitial space in the tumor. This accumulation of LPA would be sufficient to play a major role in the pathophysiology of ovarian cancer. However, the effects of the high levels of LPA present in ovarian cancer patients are amplified by aberrations in the response of ovarian cancer cells to LPA. The expression of LPA receptors on ovarian cancer cells is markedly different from that on normal ovarian epithelial cells resulting in increased responsiveness to LPA [Goetzl et al., 1999; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Further, unusual splice variants of LPA receptors, selectively linking to processes such as production of neovascularizing factors, may be present in ovarian cancer cells [Huang et al., 2004]. Finally, additional genetic aberrations in ovarian cancer cells such as amplification and mutation of multiple components of the phosphatidylinositol 3 kinase pathway sensitize ovarian cancer cells to downstream signaling effects of LPA [Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. As for changes in LPA production and metabolism, the genetic mechanisms underlying these processes remain to be determined. Nevertheless, taken together, LPA production or action appears well justified as a target for therapy in ovarian cancer.

Although freshly isolated ovarian cancer cell preparations and ovarian cancer cell lines are consistently responsive to LPA, normal ovarian surface epithelial cells (OSE) do not demonstrate significant responses to LPA [Goetzl et al., 1999; Eder et al., 2000; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. In contrast, OSE demonstrate marked responses to S1P, whereas ovarian cancer cells demonstrate more limited responses to S1P [Goetzl et al., 1999]. Consistent with the shift from S1P-dependence to LPA-dependence, ovarian cancer cell lines express markedly increased levels of mRNA

and protein for the LPA2 and -3 receptors and decreased levels of mRNA and protein for S1P receptors [Goetzl et al., 1999] as compared to OSE which have very low levels of LPA2 and -3. There are no consistent changes in levels of LPA1 receptors between OSE and ovarian cancer cell lines. However, LPA1 may function as a negative LPA receptor, as overexpression of LPA1 results in decreased proliferation as a consequence of increased apoptosis [Furui et al., 1999]. Analysis of ovarian cancer cells directly from the patient paints a similar picture in that LPA1 levels are not different from those in normal epithelium and LPA2 and -3 are each elevated in approximately 40% of tumors with a significant overlap resulting in increased expression of LPA receptors in approximately 60% of ovarian cancers [Fang et al., 2002]. Thus acquisition of expression of LPA2 and -3 during transformation leads to increased responses to LPA potentially contributing to the pathophysiology of ovarian cancer. LPA4 receptors are reported to expressed in ovary, however, the initial report did not indicate whether this was in stroma or epithelium [Noguchi et al., 2003]. As assessed by QPCR, ovarian cancer cells have low to absent levels of LPA4.

Ovarian cancer cells contain an unusual variant of LPA2 with an intracellular extension [Huang et al., 2004]. This extension destroys the PDZ binding site in LPA2 that may have important functional consequences. The variant exhibits increased ability to link to production of active VEGF, an important permeability and neovascularizing factor [Hu et al., 2001; Huang et al., 2004]. LPA couples efficiently to VEGF production potentially contributing to the elevated VEGF levels in ovarian cancer ascites [Zebrowski et al., 1999; Hu et al., 2001]. Further, although LPA1 and -3 appear able to couple to the production of IL8 and IL6 neovasularization factors, LPA2 appears to most efficient in mediating production of these factors [Schwartz et al., 2001; Fang et al., 2003]. As IL8 production is dependent on NfκB mediated activation of the IL8 promoter, it appears likely that LPA2 couples most efficiently to activation of NfκB [Fang et al., 2003]. In ovarian cancer, VEGF appears to play a major role in the production of ascites whereas IL8 production appears to regulate aggressiveness and potentially neovascularization. The effects of the increased ability of LPA2 to couple to these pathways suggest that inhibitors targeting the LPA2 receptor may selectively alter neovascularization.

Expression of the ovarian cancer specific LPA2 variant in the stroma (the promoter used does not express or is expressed at low levels in the epithelial cells of the ovary, the most frequent precursor for ovarian cancer) of transgenic murine ovaries results in constitutive production of active VEGF-A again supportive a selective role of LPA2 in production of neovascularizing factors [Huang et al., 2004]. In addition expression of the ovarian cancer specific LPA2 variant increases the production of urokinase (uPA), compatible with previous reports showing that these factors are regulated by LPA in ovarian cancer cells [Pustilnik et al., 1999; Huang et al., 2004]. This was accompanied by increased expression of VEGF receptors and decreased production of type 2 PA inhibitor. Together, this suggests that activation of the LPA2 receptor aberrantly expressed in ovarian cancer cells may contribute to neovascularization and also result in ovarian cancer cells being responsive to VEGF. If similar processes affect other neovascularizing factors such as IL6 and IL8 activation of LPA receptors may stimulate a number of aberrant autocrine loops.

LPA appears to activate a number of additional feed-forward autocrine signaling loops. For example, LPA can induce the production of endothelin, a potent activator of ovarian cancer cells [Chua et al., 1998]. Indeed, endothelin receptor isoforms appear to be aberrantly expressed in ovarian cancer. Alternatively, the effects of LPA on ovarian cancer cells may be due to LPA-induced production or processing of growth factors, which in turn activate cognate cell surface receptors. As described above, LPA induces the activation of a number of tyrosine kinase linked growth factor receptors including multiple members of the human EGF receptor (HER) family, and the platelet-derived growth factor receptor. LPA-induced processing of HB-EGF and PDGF, which, in turn, activates members of the HER family and PDGFR respectively. LPA also induces activation of Src either through activation of tyrosine kinase linked receptors or through other as yet unclear mechanisms. As src and EGFR family members are overexpressed in ovarian cancers, this may amplify the effects of LPA. Indeed, we have demonstrated that LPA induces tyrosine-phosphorylation of multiple members of the HER family and of Src in ovarian cancer cells,

compatible with LPA inducing proliferation through the increased production or action of other growth factors which activate members of the HER or PDGFR family [Xu et al., 1995; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Nevertheless, the relative role of the direct and indirect activation of ovarian cancer cells by LPA in the pathophysiology of ovarian cancer remains to be elucidated.

Ovarian cancer cells may also have aberrant responses to other lysophospholipids. As indicated above, there are aberrations in the expression of both LPA and S1P receptors that could alter the responsiveness of ovarian cancer cells [Goetzl et al., 1999]. In at least one model system, S1P can transdominantly inhibit responses to LPA suggesting that the shift in receptor selectivity from S1P in normal ovarian epithelial cells to LPA in ovarian cancer cells may have functional consequences in addition to the effects of increased LPA receptor levels [Clair et al., 2003]. The OGR1 orphan GPCR, which is overexpressed in some ovarian cancer cell lines, has been demonstrated to be a specific receptor for SPC [Xu et al., 2003]. SPC increases cytosolic calcium in ovarian cancer cells but appears to decrease cellular proliferation, questioning the role of SPC and OGR1 in transformation of ovarian epithelium [Xu et al., 1995]. LPC, LPS, and PAF can activate ovarian cancer cells, but their effects on physiological responses are unclear [Xu et al., 1995]. PA, which is both a precursor and a product of LPA metabolism, can modestly increase proliferation of ovarian cancer cells [Xu et al., 1995]. The complete spectrum of responses to the different lipids, lysolipids, and isoforms in ovarian cancer will require a thorough evaluation of the levels and responses to these moieties. Several may prove both to be functionally important and optimal targets for therapy.

The responses of ovarian cancer cells to LPA have been extensively evaluated (see Fang et al., 2002; Mills et al., 2002 and references therein). At concentrations to which ovarian cancer cells are exposed, LPA can modestly increase the proliferation of ovarian cancer cells. Its pathophysiological role in ovarian cancer may link more tightly to the metastatic cascade than to cell growth as in addition to the effects on the production of neovascularizing factors described above, it increases invasiveness through altering cytosekeletal organization, increasing cellular motility, increasing production and action of urokinase plasminogen activator (uPA), and activity of metalloproteinases (MMP2 and MMP9), all critical components in the metastatic cascade. For cells to metastasize, they must detach from their underlying matrix and move to a new site. The vast majority of tumor cells die from anoikis, a form of apoptosis. LPA is highly protective from both apoptosis and anoikis. The ability of LPA to prevent apoptosis may also contribute to the poor outcome in the disease through induced resistance to the main drug used in ovarian cancer cisplatin [Fang et al., 2002; Mills et al., 2002].

The mechanisms by which LPA mediates it functional effects on ovarian cancer cells are only beginning to be delineated. In ovarian cancer cells, LPA through binding to its cognate receptors induces increases in cytosolic free calcium, Rac, Rho, and Rock activation, activation of phospholipase C and D, as well as activation of tyrosine kinases and the PI3K and Ras/MAP signaling cascades [Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. These in turn lead to activation of NFκB, AP1, and AP2. The roles of specific signaling pathways in specific responses to LPA remain to be delineated.

Almost half of all drugs in current use target members of the GPCR family of receptors making the Edg family of LPA receptors attractive targets for therapeutic development. By analyzing the structure–function relationships of the Edg receptors using yeast, insect and mammalian cells, we and others are beginning to develop receptor selective agonists and antagonists for LPA1, -2, and -3 [Fang et al., 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Feng et al., 2003]. As LPA1 may be a negative growth regulator, agonists of LPA1 may decrease viability and growth of ovarian cancer cells. However, as LPA1 can mediate cellular motility, this could come at the consequence of increased tumor spread. In contrast, LPA2 and -3 can increase motility, production of neovascularizing factors and induce proliferation, respectively, suggesting that antagonists may demonstrate optimal activity. Further, LPA2 is implicated in the production of neovascularizing factors and in particular IL8, which may define the aggressiveness of ovarian cancer [Fang et al., 2003]. LPA3 is particularly appealing as it is selectively activated by the subtype of LPA (with unsaturated fatty acyl chains) that

are found at high levels in ascites from ovarian cancer patients [Bandoh et al., 1999; Xiao et al., 2001; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Indeed, LPA subtypes with unsaturated fatty acyl chains are highly active on ovarian cancer cells. LPA receptors are expressed by multiple cell lineages including fibroblasts, platelets, and brain cells [Fang et al., 2002]. However, the observation that LPA3 is selectively elevated in ovarian cancer cells and that ovarian cancer cells are particularly responsive to specific species of LPA (polyunsaturated fatty acyl chains) suggests that inhibitors based on these forms of LPA may demonstrate specificity for ovarian cancer cells. Taken together, ovarian cancer appears to be driven through the production and action of LPA. Thus inhibitors of LPA production or action may provide an effective approach to the therapy of this disease.

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LYSOPHOSPHATIDIC ACID LIPIDOMICS REVEALS AN INTERACTION BETWEEN PYRUVATE KINASE AND CLATHRIN HEAVY CHAIN/HSP70*

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Running title: Pyruvate kinase-LPA interaction

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Lysophosphatidic acid (LPA; 1-acyl-2 hydroxy-*sn*-glycero 3-phosphate) is a pleiotropic lipid signalling molecule that evokes a broad array of cellular responses including proliferation, tumor cell invasion, neurite retraction, cytoskeletal rearrangements and smooth muscle contraction. Generally, lysophosphatidic acid triggers physiological responses through interaction with specific plasma membrane receptors called LPA(1-4). There is however increasing evidence in support of intracellular proteins that interact with LPA. We employed Affi-Gel-immobilized LPA to isolate cytoplasmic proteins that interact with this lysophospholipid. Among several candidate proteins retained by this affinity matrix, pyruvate kinase, clathrin heavy chain and heat shock protein 70 (Hsp70) were identified by mass spectrometry. Isothermal titration calorimetry shows that pyruvate kinase contains two binding sites for LPA (K_a ~10^7 M^{-1} and K_a ~ 10^6 M^{-1}). Furthermore, LPA dissociates enzymatically active pyruvate kinase tetramers into inactive dimers, and is maximally active at concentrations close to its critical micelle concentration. These effects were not mimicked by other lysophospholipids. Co-immunoprecipitation experiments show that pyruvate kinase interacts with clathrin and Hsp70, and confocal imaging revealed co-localization between clathrin and pyruvate kinase in the perinuclear region of cells. Our data suggest that pyruvate kinase partly exists in complex with clathrin at subcellular membranous areas, and that locally increased LPA levels can trigger inactivation of the metabolic enzyme.

Lysophosphatidic acid (LPA; 1-acyl-2 hydroxy-*sn*-glycero 3-phosphate) is one of the simplest natural phospholipids and a precursor in the *de novo* biosynthesis of phospholipids. In recent years, it has become clear that this lysophospholipid mediates various cellular processes, such as proliferation, differentiation, survival, migration, adhesion, invasion, and morphogenesis (1-3).

Blood serum LPA is secreted by activated platelets, activated adipocytes, neuronal cells, and other cell types (4-6). Although mechanisms of LPA synthesis in individual cell types remain to be elucidated, serum LPA is produced by multiple enzymatic pathways that involve monoacylglycerol kinase, phospholipase A1, secretory phospholipase A2, and lysophospholipase D (lysoPLD), including autotaxin (5;7;8).

Extracellular LPA evokes biological responses that are mediated through the activation of four G protein-coupled receptors (LPA1-4) (9). In serum, LPA binds to gelsolin (10) and albumin (the main extracellular LPA binding protein) with a nanomolar range affinity

for the phospholipid and a stoichiometry of about 3 mol of LPA/mol of albumin (11;12). Although important progress has been made in the understanding of the extracellular effects of LPA and its role in disease states such as cancer progression and atherosclerosis, the role of intracellular LPA has only recently been recognized. The role of intracellular LPA as an intermediate in the early steps of phospholipid biosynthesis is well established. This process occurs in the endoplasmatic reticulum, where LPA is formed from glycerol-3-phosphate by acylCoA and then further acylated to phosphatidic acid (PA), the precursor of all glycerophospholipids.

Weigert et *al.* (13) showed that CtBP/BARS, a protein that is involved in Golgi tubule dynamics, is an essential component of the fission machinery operating at the Golgi tubular network. These authors found that CtBP/BARS uses acyl-CoA to selectively catalyze the acylation of LPA to PA both in pure lipidic systems and in Golgi membranes, and this reaction is essential for fission. Addition of LPA enhances the reaction and induces massive fission/defragmentation of the Golgi tubular networks whereas other lysolipids have no effect. Lipid microdomains rapidly interconverting LPA, PA and diacylglycerol, have the potential to facilitate the overall process of fission through coordinated changes in local membrane curvature. The lipid machinery probably involves other proteins. The best-characterized fission protein so far is dynamin, which acts in concert with other proteins at the neck of endocytic vesicles. The dynamin binding protein endophilin is required for the formation of endocytic vesicles and has, like CtBP/BARS, LPA acyltransferase activity (14).

Intracellularly, LPA binds to fatty acid binding proteins (FABP). The liver-type FABP (11) exhibits micromolar range affinity for LPA and allows the transport of mitochondrial LPA to microsomes in order to be acylated into PA (15). Gelsolin could also play an important role in LPA binding and transport. Cytoplasmic gelsolin binds, severs and caps actin filaments. LPA as well as phosphoinositides containing D3- and D4-phosphate group bind to gelsolin, promoting actin filament uncapping and thus providing sites for actin assembly (16). Recently, a role for intracellular LPA as a signaling molecule has been suggested by the finding that LPA (at relatively high doses) can compete with a synthetic ligand for binding to the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ). Extracellular LPA induces PPARγ-mediated reporter gene transcription in an LPA receptor-independent manner. It was also shown that LPA enhanced expression of endogenous CD36 through PPARγ. CD36 is involved in internalizing oxidized LDL in monocytes leading to formation of foam cells that are rich in cholesterolesters (17). This is an early event in the formation of atherosclerotic plaques and Zhang and colleagues have indeed demonstrated that LPA activation of PPARγ can lead to accumulation of cells in the arterial wall (18). Intracellular LPA is also a potent regulator of the subfamily of mechano-gated K_{2P} channels comprising TREK-1, TREK-2 and TRAAK. Channel mechano-sensitivity is drastically and reversibly altered by intracellular LPA (19).

A recurring theme in studies on lipid mediators is their possible signaling role inside the cell. In order to identify intracellular LPA binding proteins we designed an affinity matrix consisting of a LPA analog covalently linked to Affi-Gel 10. After incubation of cell extracts with this affinity matrix, several LPA-binding proteins were isolated and identified by mass spectrometry. As a proof-of-principle, validation and characterization of one of these LPA-binding proteins, pyruvate kinase, is described.

Experimental procedures

Reagents-Rabbit muscle pyruvate kinase was purchased from MP Biomedicals, Inc. (Aurora, USA). Lactate dehydrogenase was from Calbiochem (San Diego, USA). Phosphoenolpyruvate, ADP, NADH, lysophosphatidylcholine, lysophosphatidylserine, lysophosphatidyinositol and clathrin heavy chain antibody were all purchased from Sigma (St Louis, USA). LPA (18:1), LPA (14:0) and LPA (6:0) were from Avanti polar lipids (Alabaster, USA). Alexa 594-conjugated goat anti-mouse and Alexa 488-conjugated rabbit anti-goat were obtained from Molecular Probes (Eugene, USA). Molecular mass markers for SDS-PAGE were from Bio-Rad (Hercules,USA). Phenylmethylsulfonyl fluoride was from Serva (Heidelberg, Germany). Other protease inhibitors were from Amersham Biosciences (Uppsala, Sweden). Antibodies against pyruvate kinase and Hsp70 were obtained from Abcam (Cambridge, UK).

Synthesis of tethered LPA and PA ligands and preparation of the affinity matrices-An 11-aminoundecanoyl-linked LPA analog was synthesized for direct immobilization of LPA on Affi-Gel-10 beads (20;21). The synthesis started with the phosphorylation of *rac*-solketal using dimethyl chlorophosphate in the presence of tertiair potassium butanol (*t*-BuOK) (22). Hydrolysis of the acetal produced a diol, which was esterified with benzyloxycarbonyl (Cbz) protected 11-aminoundecanoic acid selectively at *sn*-1 position at 0°C using dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (DCC/DMAP) to produce the protected 11-aminoundecanoyl-LPA analog (23). Trimethylsilyl bromide/methanol (TMSBr/MeOH) treatment resulted in elimination of the dimethyl phosphate and Cbz amino protecting groups at the same time, generating the LPA-NH_2, (24) which was then coupled to Affi-Gel-10 in anhydrous methanol with a trace of triethylamine (TEA). Upon addition of, the NHS was displaced and a stable amide bond was formed (Fig. 1). An excess of Affi-Gel-10 was employed to completely consume the aminoacyl LPA and to produce a loading of approximately 1 – 2 μmol/ml resin. Following an overnight reaction with ligand, the reaction mixture was treated with a 50-fold excess of 2-aminoethanol to cap any remaining reactive ester groups. The reaction was monitored by increased UV absorption at 280 nm due to the NHS release during the coupling to ligand. As the fatty acid at the *sn*-1 position anchors LPA to the Affi-Gel 10 resin, the head group of the lysophospholipid is expected to be exposed to solvent and thus available for binding.

To prepare the control aminoacyl-linked PA affinity resin, intermediate was esterified with 6-carbobenzyloxyaminocaproic acid (DCC, DMAP, 0°C) selectively at the *sn*-1 position, and then with hexanoic acid at *sn*-2 position (DCC, DMAP, 20°C). The resulting protected aminoacyl-PA analog was deprotected as described above for the LPA analog. Coupling of PA with Affi-Gel-10 proceeded as described for the aminoacyl-LPA analog. An additional control resin with no ligand was prepared by reaction of Affi-Gel 10 with excess 2-aminoethanol.

Cell culture and fractionation and lipid affinity chromatography-MCF7 cells were maintained in 1/1 (v/v) F12 nutrient mixture (Ham, Invitrogen) with L-glutamin and Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen, Merelbeke, Belgium), 100 U/ml penicillin and 0.1 mg/ml streptomycin. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum, 0.05% L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Cells were washed twice with ice cold phosphate-buffered saline (PBS)((2.7 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) and 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium ortho-vanadate, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Roche, Basel, Switzerland). The mixture was sonified and centrifuged at 20,000 × *g* in a micro centrifuge for 10 min at 4°C to remove insoluble cell debris. LPA beads, stored in 20% ethanol, were equilibrated with three washes in lysis buffer. In a typical binding reaction, 1 ml cytosol (containing 2 mg total protein) was mixed with 5 μl of settled beads on ice. The tubes were put in a rotator at 4° C for 2 h. Following binding, the beads were quickly washed three times with lysis buffer and resuspended in Laemmli sample buffer, and bound proteins were analyzed by SDS-PAGE.

Identification of Polypeptide Bands by Q-TOF-Coomassie-stained gel bands were cut out of the gel and transferred to a Biopure®Eppendorf tube (Eppendorf AG, Hamburg, Germany). After washing with water (HPLC graded, Baker Malinckrodt B.V., Amsterdam, the Netherlands), 50% (v/v) acetonitrile (Baker Malinckrodt B.V.) in water and 100% acetonitrile, they were submerged in 50 mM ammonium bicarbonate at pH 8.0 and digested using trypsin. The digestion proceeded overnight at 37°C and was stopped by acidification through the addition of formic acid. After centrifugation, the peptide mixture was transferred to a new Eppendorf tube, dried and re-dissolved in 20 μl of 0.1% formic acid in 2/98 (v/v) acetonitrile/water (solvent A). 10 μl of this peptide mixture was applied for nano-LC-MS/MS analysis on a Waters CapLC (Waters Corporation, Milford, MA, USA) on-line connected to a Q-TOF1 mass spectrometer (Waters-Micromass UK Limited, Cheshire, UK). The sample was first trapped on a trapping column (PepMap™ C18 column, 0.3 mm I.D. x 5mm, Dionex (Amsterdam, The Netherlands)). After back-flushing from the trapping column, the sample was loaded on a 75 μm I.D. x 150 mm reverse-phase column (PepMap™ C18, Dionex (Amsterdam, The Netherlands)).

Following a 5 min wash with solvent A, the peptides were eluted with a linear gradient of 4% solvent B (0.1% FA in water/acetonitrile (3/7, v/v)) increase per minute at a constant flow rate of 5 µl/min. Using data dependent acquisition, doubly or triply charged ions with intensities above threshold 40 were selected for fragmentation. During MS/MS analysis, a cone voltage of 30 V and a scan time of 1 s with an inter scan time of 0.07 s was used. Data acquisition was initiated 15 min following the start of the gradient. The fragmentation spectra were converted to peaklist (pkl) files using the Masslynx® software (version 3.5, Micromass) and were searched using the MASCOT database search engine (http://www.matrixscience.com) against the SwissProt database with restriction to human proteins. Peptide mass tolerance was set at 0.25 Da and peptide fragment mass tolerance at 0.3 Da, with the ESI-QUAD-TOF as selected instrument for peptide fragmentation rules. Variable modifications were set to methionine oxidation, pyroglutamate formation of amino terminal glutamine, deamidation (both for asparagine and glutamine), acetylation of the N-terminus and propionamide modification of cysteines.

Isothermal titration calorimetry-
Microcalorimetric titration measurements were performed in a Microcal Omega isothermal titration calorimeter (Microcal Inc., Northampton, MA). All solutions were degassed under vacuum prior to use. In a typical experiment, 1.33 ml of 60 µM pyruvate kinase in 20 mM Tris-HCl, 150mM NaCl, pH 7.5 was titrated by 20 x 15 µl injections with 400 µM LPA. During titration, the injection syringe was rotated at 250 rpm. Time between injections was set at 5 min. In a blank experiment, heat evolving from dilution was measured by injecting the LPA solution into the sample cell filled with 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. This heat of dilution was subtracted from the corresponding LPA binding data of pyruvate kinase. Data were integrated and fitted to an appropriate binding model using the ORIGIN software supplied by Microcal Inc.

Enzyme activity measurements-The enzyme activity was measured at 25 °C in a coupled system with lactate dehydrogenase (LDH) and NADH (25). Standard assays were performed in the following medium: 1.5 mM ADP, 120 mM KCl, 62 mM MgSO4, 1.5 mM phosphoenolpyruvate, 0.22 mM NADH and 4 units/mL LDH (which represented a large excess over pyruvate kinase) in 50 mM Imidazole-HCl buffer at pH 7.6. The reaction was initiated by the addtion of 5 µL of pyruvate kinase to the cuvette containing 0.5 ml of the above medium and was monitored by the decrease in absorbance at 340 nm for 5 min. The rate of reaction was measured from the initial linear region of the curve. Pyruvate kinase activity in the presence of different lysophospholipids was determined after 30 min incubation at room temperature. Pyruvate kinase concentration was determined by absorption measurements at 280 nm, using an extinction of 0.54 $ml.mg^{-1}.cm^{-1}$ (26).

Gel filtration chromatography-This was performed on a Waters Advanced Protein Purification System apparatus, using a precalibrated superdex 200 PG 10/60 column (60 x 1.0 cm), and 0.05 M Tris-HCl, 0.15 M NaCl, pH 8.0 as running buffer. Reference markers were Blue dextran (MW 2,000,000), Ferritin (MW 450,000), Catalase (MW 240,000), Lactate dehydrogenase (MW 140,000), Bovine serum albumin (MW 66,000), Ovalbumin (MW 45,000), Chymotrypsinogen A (MW 28,000), Myoglobuline (MW 17,000), Vitamin B12 (MW 1350) and Dinitrophenylasparagine (MW 298).

Nondenaturing polyacrylamide gelelectrophoresis-Pyruvate kinase was incubated with or without increasing amounts of LPA (18:1) on ice for 30 minutes. Mixtures were loaded onto a 6% nondenaturing polyacrylamide gel, run at 4°C for 2 h at 90 V in a Tris-glycine buffer pH 8.6. Pyruvate kinase was visualized by Coomassie staining.

Circular dichroism measurements-Circular dichroism measurements were carried out at room temperature on a Jasco 710 spectropolarimeter between 190 and 260 nm in quartz cells with a path length of 0.1 cm. Sixteen spectra were recorded and averaged. Pyruvate kinase was diluted to a concentration of 0.1 mg ml^{-1} in a 10 mM sodium phosphate buffer. The CD spectra of pyruvate kinase in the presence of different lysophospholipids were recorded after 30 min incubation at room temperature of the enzyme with lipids at a molar ratio of 1/100. The spectra were corrected for minor contributions of lipids by subtracting the measured spectra of lipids alone. The secondary structure of pyruvate kinase was determined by curve fitting to reference protein spectra using the CDNN program (27). The helicity of pyruvate kinase was determined from the mean residue ellipticity [θ] at 222 nm (28).

Immunoprecipitation experiments-Cells were washed twice in ice-cold PBS and lysed in PBS supplemented with 0.5% Nonidet P-40, 10 mM sodium fluoride, 8 mM sodium-β-glycerophosphate and a protease inhibitor cocktail mix (Roche Diagnostics). Cells were disrupted by sonication and centrifugation at 4° C for 10 minutes (20,000 × *g*). We incubated 1 mg of proteins overnight with affinity-purified antibodies against either pyruvate kinase, Hsp70 or clathrin heavy chain and subsequently with protein G-Sepharose (Amersham Pharmacia Biotech,Uppsala,Sweden) for 4 h. Pellets were washed three times with lysis buffer, boiled for 5 min in Laemmli sample buffer and fractionated by SDS-PAGE followed by western blotting. Proteins were analysed by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden).

Fluorescence Microscopy and Immunocytochemistry-The cells were fixed with 3% para-formaldehyde, permeabilized in 0.1% Triton X-100, washed in PBS, incubated with primary antibody against pyruvate kinase for 1 h at 37°C. After several washes with PBS, the cells were incubated with Alexa 488-conjugated donkey anti-goat secondary antibody 30 min at room temperature. Clathrin staining was performed with a monoclonal antibody against clathrin heavy chain and Alexa 594-conjugated goat anti-mouse as secondary antibody. Photographs were taken with a Zeiss model LSM410 inverted on the basis of a Zeiss Axiovert 100 microscope (Carl Zeiss B.V., Sliedrecht, the Netherlands).

Miscellaneous- Protein concentrations were determined by the method of Bradford (29) using bovine serum albumin as a standard. We carried out SDS-PAGE according to Matsudaira and Burgess (30). Western blot was done by the method of Towbin *et al.* (31). The fluorescent probe 2-(p-toluidinyl)-naphtalene-6-sodium sulfonate (TNS) was used to determine the critical micelle concentration of LPA 18:1 and LPA 14:1. Upon mixing of TNS with the lysophosholipid, a large increase in fluorescence is observed if the lipid exceeds the critical micelle concentration (32).

Results

Synthesis of LPA and PA affinity matrices

To enable the isolation of novel LPA-binding proteins, we designed immobilized LPA and PA resins as shown in Figure 1 (for PA resin, see also (33)). An 11-aminoundecanoyl-linked LPA analog (**4**) was synthesized for direct immobilization of LPA on Affi-Gel-10 beads (20;21). LPA-NH_2 (**4**), (24) was coupled to Affi-Gel-10 in anhydrous methanol with a trace of triethylamine (TEA). The anhydrous organic solvent was used to minimize hydrolysis of NHS esters that occurs in aqueous solutions. Upon addition of (**4**), the NHS was displaced and a stable amide bond was formed (Fig. 1). An excess of Affi-Gel-10 was employed to completely consume the aminoacyl LPA (**4**) and to produce a loading of approximately 1 – 2 μmol/ml resin. This loading minimizes premature clogging of the resin by nonspecific protein adsorption. Following an overnight reaction with ligand, the reaction mixture was treated with a 50-fold excess of 2-aminoethanol to cap any remaining reactive ester groups. The reaction was monitored by increased UV absorption at 280 nm due to the NHS release during the coupling to ligand (**4**). As the fatty acid at the *sn*-1 position anchors LPA to the Affi-Gel 10 resin, the head group of the lysophospholipid is expected to be exposed to solvent and thus available for binding.

To prepare the control aminoacyl-linked PA affinity resin the aminoacyl PA analog (**7**) was coupled with Affi-Gel-10 as described for the aminoacyl-LPA analog. An additional control resin with no ligand was prepared by reaction of Affi-Gel 10 with excess 2-aminoethanol.

Isolation and identification of Cytosolic Proteins that interact with LPA

We used HEK293T and MCF-7 cells as a source of material for isolation of LPA-binding proteins. After incubation with the LPA beads, unbound material was removed with several washes. The bound proteins were separated by SDS-PAGE and visualized by Coomassie staining (Fig.2). It should be noted that the protein pattern of MCF-7 cells is very similar to the pattern of HEK293T cells. The identity of LPA-binding polypeptides was established by Q-TOF mass spectrometry. In total, 12 polypeptides were identified. Among the candidate LPA interacting proteins, we further examined pyruvate kinase, clathrin heavy chain and Heat Shock Protein 70 (Hsp70) because of their relatively high abundance. The identity of pyruvate kinase, clathrin heavy chain and Hsp70 were also confirmed by western blotting (data not shown). To validate our method,

commercially available pyruvate kinase M1 was further characterized with respect to its lipid-binding properties using isothermal titration calorimetry (ITC).

Binding of rabbit muscle pyruvate kinase to lysophospholipids

The interaction of pyruvate kinase with lysophosphatidic acid was studied by ITC. Fig.3 shows a typical experiment in which LPA solution was injected into the pyruvate kinase solution in the sample cell. Fig. 3A shows the raw data and Fig. 3B shows the integrated released heat as a function of the molar ratio of LPA/pyruvate kinase. After subtraction of the control (injection of lysophosphatidic acid into buffer) the binding isotherm clearly shows an exothermic process with two binding sites. The solid line represents the best fit of the data. The highest affinity binding site shows a K_a value of $\sim 10^7$ M^{-1} with a stoichiometry of ~0.25 mole LPA/mole pyruvate kinase and a reaction enthalpy of - 9 kcal/mol. The second binding site has a K_a value of $\sim 10^6$ M^{-1} with a stoichiometry of ~ 0.75 mole LPA/mole pyruvate kinase and an enthalpy change of - 4 kcal/mol. As pyruvate kinase exists as a tetramer in solution (34). These data suggest that one tetramer of pyruvate kinase binds four mol LPA. Titration of pyruvate kinase with lysophosphatidylcholine (LPC) or lysophosphatidylserine (LPS) showed no specific heat release after subtraction of the control (injection of the lysophospholipid into buffer) (data not shown).

Binding of pyruvate kinase to LPA is direct and specific

To examine the specificity of pyruvate kinase-LPA and clathrin-LPA interaction we incubated MCF-7 cell lysate with Affi-Gel 10 beads coupled to LPA, PA or phosphatidylinositol 4,5 bisphosphate (PIP_2). Western blot analysis showed that pyruvate kinase was strongly enriched by the LPA matrix, weak interaction occurred with the PA matrix, and no retention was observed on the PIP_2 matrix. Clathrin was also retained by the LPA beads (Fig.4A) but there was no significant signal following incubation with the PA beads. In both cases there was no interaction with Affi-Gel beads. Furthermore, if binding of pyruvate kinase to immobilized LPA is specific, then soluble LPA should compete and reduce the interaction between immobilized LPA and pyruvate kinase. We competed for binding of pyruvate kinase to Affi-Gel-coupled LPA with hexanoyl LPA (C6:0) or oleoyl LPA (18:1). When pyruvate kinase was preincubated with monomeric LPA (C6:0), no competition was noticed (Fig 4B, lane 9-12), whereas preincubation with increasing concentrations LPA (C18:1) gradually abolished binding of pyruvate kinase to the LPA matrix (Fig. 4B, lanes 2-8).

Effect of lysophospholipids on pyruvate kinase activity

The activity of pyruvate kinase was examined in the presence of hexanoyl LPA (6:0), myristoyl LPA (14:0) or oleoyl LPA (18:1) (Fig 5A). No effect was seen with hexanoyl LPA (6:0) over the entire concentration range (0-600 μM). With LPA (14:0) and LPA (18:1), the activity was reduced at concentrations higher than 400 μM and 50 μM, respectively. These concentrations are approximately the critical micellar concentration (CMC) of LPA (14:0) and LPA (18:1). Indeed, measurements with 2-(p-toluidinyl)-naphtalene-6-sodium sulfonate (TNS) yielded CMC values of ~370 μM and ~50 μM for LPA (14:0) and LPA (18:1), respectively (data not shown). At concentrations above the CMC of LPA (18:1), pyruvate kinase activity was almost completely abolished.

We also measured the activity of pyruvate kinase in the presence of different lysophospholipids (Fig. 5B). Lysophosphatidylcholine (LPC) and lysophosphatidylserine (LPS) had no significant effect on pyruvate kinase activity, even at lipid concentrations exceeding their CMC. Lysophosphatidylinositol (LPI) only showed a minor effect at higher concentrations. We therefore conclude that LPA inhibits pyruvate kinase activity more specifically than other lysophospholipids.

Strong binding of LPA to pyruvate kinase at concentrations close to its CMC was also noticed following non-denaturing gel electrophoresis (Fig. 5C). A sharp decrease in lipid-free pyruvate configuration was observed at LPA concentrations of 50 μM and higher.

LPA dissociates active pyruvate kinase tetramers into inactive dimers

It has been documented that mammalian pyruvate kinase switches between a less active dimeric form and a highly active tetrameric form (34). We investigated by gel filtration

chromatography if the decrease in activity of pyruvate kinase upon incubation with LPA (18:1) was due to conversion of the tetrameric form into a dimeric form. Elution profiles of pyruvate kinase in buffer before and after incubation with lysophospholipids at a 1/100 pyruvate kinase/lipid molar ratio on a calibrated Superdex 200 PG column are shown in Fig. 6. Pyruvate kinase in buffer elutes from the column as a tetramer with an apparent molecular weight of 233 kDa. Upon incubation with LPA (18:1) at a 1/100 molar ratio, two fractions eluted from the column with apparent molecular weights of 233 and 100 kDa, respectively. The second peak most likely corresponds with a dimer of pyruvate kinase. Pyruvate kinase activity in the top fraction of both peaks was measured: the first fraction retains full activity whereas the second peak was completely devoid of activity (data not shown). This effect was again specific for LPA since LPC, LPS or LPI promoted no dissociation of pyruvate kinase at molar ratios similar to LPA.

Effect of lysophospholipids on the secondary structure of pyruvate kinase

The circular dichroism (CD) spectra of pyruvate kinase in phosphate buffer with or without LPA, LPC, LPS or LPI are shown in Fig. 7. The CD spectrum of pyruvate kinase in phosphate buffer is indicative of an alpha helical structure (35) (characteristic minima at 208 and 222 nm). Upon incubation with LPA the alpha helicity is increased from ~32 to ~ 36% at the expense of random coil. Incubation with LPC or LPS did not affect the secondary structure of pyruvate kinase, whereas LPI also slightly increased the α helical structure These data suggests that LPA does not unfold the enzyme although it induces dissociation of the tetrameric configuration into an inactive dimeric form.

Pyruvate kinase Forms a Complex with clathrin and Hsp70

Hsp70, clathrin heavy chain and pyruvate kinase were identified in this study as possible LPA binding proteins. Pyruvate kinase has been reported to interact directly with HERC1 (36). HERC1 is a giant protein of almost 5000 amino acids long, and is localized in inner cell membranes such as the Golgi apparatus and purportedly plays a role in intracellular traffic (37). Because HERC1 forms a cytoplasmic ternary complex with clathrin and Hsp70 (38) we investigated whether pyruvate kinase could form a complex with clathrin and Hsp 70 by co-immunoprecipitation assays. Immunoprecipitation of pyruvate kinase from MCF-7 cells with a polyclonal antibody followed by western blotting showed co-precipitation of clathrin but not of Hsp70 (Fig. 8A, lane 1). Conversely, clathrin heavy chain immunoprecipitation with a monoclonal antibody revealed that both Hsp70 and pyruvate kinase are present in the complex (Fig. 8B, lane 1). These findings suggest that clathrin heavy chain and Hsp70 may be retained by immobilized LPA through interaction with pyruvate kinase, although it does not exclude a direct interaction between Hsp70 or clathrin with LPA.

Clathrin and pyruvate kinase partly co-localize in MCF-7 cells

The subcellular localization of endogenous clathrin and pyruvate kinase was studied by indirect immunofluorescence and confocal microscopy in MCF-7 cells (Fig. 9). Both proteins displayed a punctate staining. Whereas pyruvate kinase appeared distributed uniformly in the cytoplasm with occasional enrichment in particular areas, clathrin showed enrichment in the perinuclear area in addition to a characteristic dot-like distribution in the cytoplasm. This punctate staining may suggest that these proteins interact with intracellular membranous structures (36;39). Of note, superposition of both images showed a partial overlap in distinct areas of the cell, particularly in the perinuclear region, with occasional co-localization in isolated cytoplasmic membranous compartments.

Discussion

Relatively few intracellular binding partners for LPA have been recognized in recent years. Proteins identified so far include CtBP/BARS (13), L-FABP (11) and gelsolin (10;16). LPA is also a ligand for PPARγ (17) and was acknowledged more recently as a regulator of mechano-gated K2P channels comprising TREK-1, TREK-2 and TRAAK (19).

For easier isolation and purification of novel LPA-binding proteins, we used a solid phase approach involving Affi-Gel-immobilized LPA. Here we describe the isolation of LPA binding proteins from representative mammalian

cells coupled to identification by mass spectrometry. Although several proteins were identified, their individual binding characteristics and affinity for the phosholipid have not been determined in each case. In addition our approach does not rule out the possibility that some proteins bind indirectly to the LPA matrix through interaction with another polypeptide present in the sample. In fact, the association reported here between pyruvate kinase M (the M1 and M2 isozyms) and clathrin heavy chain/Hsp70 may well represent such an example.

We focused on pyruvate kinase given the relatively high abundance of peptides that were identified (this is also evident from the Coomassie stained gel in Fig. 2). To investigate if pyruvate kinase can bind directly to LPA, the interaction was measured in solution by ITC. The LPA/pyruvate kinase interaction proved to be an exothermic process with an affinity in the micromolar range. A number of reports have shown that pyruvate kinase and other glycolytic proteins can reversibly associate with subcellular membrane structures (reviewed in (40)). Local regulation of the glycolysis pathway in a cell and ATP compartmentation are hypothesized to be coupled to the function of organelles within the cell. Several studies have demonstrated that glycolytic enzymes (phosphoglycerate kinase, aldolase, phosphofructokinase, lactate dehydrogenase, glucose-3-phosphate dehydrogenase, hexokinase) can associate in vitro with phospholipid bilayers (41;42). Most of the studied glycolytic enzymes are inactivated upon interaction with membranes or phospholipids (40;43-46). However, Dabrowska et al. (41) demonstrated that pyruvate kinase activity increases upon interaction with certain phospholipids, especially with phosphatidylserine incorporated in liposomes. Phosphatidylserine is an anionic phospholipids like LPA, We therefore investigated if LPA could also affect the enzymatic activity of pyruvate kinase. Our data show that LPA (18:1) decreases pyruvate kinase activity, while other lysophospholipids (LPC, LPI and LPS) do not affect its activity. Moreover, at concentrations above the CMC of LPA, pyruvate kinase activity is lost. This is clearly demonstrated with LPA (18:1) and LPA (14:0) which have CMC values of 50 μM and 370 μM, respectively, whereas the short LPA (6:0), which does not form micelles, has no effect at all on the activity of pyruvate kinase. These results could indicate that an organized lipid structure is necessary for functional interaction between the enzyme and phospholipids. This was also claimed by Terlecki et al. (47) for lactate dehydrogenase- phosphatidylserine interaction. The mammalian M2 type pyruvate kinase isozyme can switch between a less active dimeric form and a highly active tetrameric form, which regulates the channeling of glucose carbons either to synthetic processes (dimeric form) or to glycolytic energy production (tetrameric form) (46). Tumor cells are usually characterized by an overexpression of the dimeric form leading to strong accumulation of all glycolytic phosphometabolites upstream of pyruvate kinase in the glycolysis pathway (48-50). On the other hand LPA is also known to enhance tumorigenesis by increasing cellular motility and invasiveness (2;22;51). We showed that LPA-pyruvate kinase interaction can lead to dimerization of the enzyme in vitro. Upon incubation with micellar LPA, the tetrameric pyruvate kinase dissociates into an inactive dimer as demonstrated by gel filtration chromatography. Mobility shifts observed in nondenaturing gel electrophoresis additionally suggest changes in pyruvate kinase configuration. The observed dissociation into dimers did not affect the secondary structure of the enzyme as shown in the circular dichroic measurements. This dissociation into dimers was not observed with other lysophospholipids.

HERC proteins contain one or more RCC1-like domains and a C-terminal HECT domain (homologous to E6-AP (E6-associated protein) C-terminus). RCC1 is a GEF (guanine nucleotide exchange factor) for the small GTPase and nucleocytoplasmic shuttling protein Ran. Several HERC proteins have been recognized as ubiquitin ligases (52). In order to identify proteins that interact with HERC1, Rosa et al. (37;38) used several HERC1 domains as baits in the yeast two-hybrid system. They found that HERC1 forms an ATP-dependent ternary complex with clathrin and Hsp70, and reported that the HECT domain of HERC1 interacts with M2-type pyruvate kinase. Since we identified pyruvate kinase, clathrin heavy chain and Hsp70 as possible LPA binding proteins, we checked if these proteins could form a complex. Immunoprecipitation experiments with HEK or MCF-7 cellysates indicate that pyruvate kinase is able to form a complex with clathrin. We did not however detect co-immunoprecipitation of Hsp70. This may be ascribed, in part, to overlap between protein interaction sites and polyclonal antibody recognition sites because

immunoprecipitation experiments with clathrin heavy chain antibody did demonstrate interaction between clathrin heavy chain, Hsp70 and pyruvate kinase. These findings may suggest that clathrin, Hsp70, pyruvate kinase and HERC1 can form a complex involving LPA, or that their interaction is regulated by LPA. Endogenous pyruvate kinase and clathrin proteins were shown to display a punctate cytosolic and perinuclear staining when their subcellular localization was analyzed by confocal microscopy. This presumably indicates that both proteins are associated with membranous compartments (36;39) and is in agreement with the data reporting pyruvate kinase activation by PS-containing liposomes (43). LPA might thus also play a role in tethering pyruvate kinase to intracellular membrane structures.

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***Footnotes**

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The abbreviations used are: LPA, lysophosphatidic acid; PA, phosphatidic acid; LPI, lysophosphatidyl inositol; LPS, lysophosphatidyl serine, LPC, lysophosphatidylcholine; PK, pyruvate kinase; FABP, fatty acid binding protein; Hsp70, Heat shock protein 70; ITC, isothermic titration calorimetry; SDS-PAGE, sodium dodecyl polyacrylamide gelelectrophoresis; CMC, critical micelle concentration; CD, circular dichroism; ADP, adenine diphosphate; NADH, reduced form of nicotinamide adenine dinucleotide; PBS, phosphate buffered saline

FIGURE LEGENDS

Fig. 1. Synthesis of a derivatized LPA analog and coupling to Affi-Gel-10. The ω-amino functionalized lysophosphatidic acid was coupled to *N*-hydroxysuccinimide-activated ester resin, Affi-Gel 10. The Affi-Gel 10 bead is shown as a shaded sphere.

Fig. 2. Coommassie stained polyacrylamide gel (10%) revealing proteins retained by immobilized LPA (C18:1). 2 mg cytosolic proteins (MCF-7 cells, left; HEK293T cells, right) were mixed with either control Affi-Gel beads (lane1) or LPA beads (lane 2). Proteins retained by the matrix were eluted with Laemmli sample buffer and separated by SDS-PAGE. Note the similar pattern between MCF-7 and HEK293T cells. Major bands are indicated by arrows. Molecular weight markers are shown on the left.

Fig. 3. Lysophosphatidic acid – pyruvate kinase binding measured by isothermal titration calorimetry. The top panel shows raw heat data obtained from 20 injections of LPA (C18:1) into a cell containing 60 μM rabbit muscle pyruvate kinase (see Materials and Methods). The last 6 peaks correspond to heat of dilution and show that all of pyruvate kinase is saturated with LPA (C18:1). Bottom panel: binding isotherm created by plotting areas under the peaks against the molar ratio of LPA (C18:1) added to pyruvate kinase. The fit (line) corresponds with a model of four LPA molecules per tetramer of pyruvate kinase.

Fig. 4. Specificity of clathrin and pyruvate kinase binding to LPA C18:1).
A, MCF-7 cell lysate was incubated with control Affi-Gel beads (lane 1), LPA (C18:1) beads (lane 2), PA beads (lane 3) or PIP_2 beads (lane 4). Retained proteins were separated by SDS-PAGE, blotted and probed with anti-pyruvate kinase antibody (left) or anti-clathrin heavy chain antibody (right). *B*, Purified pyruvate kinase (1 μM) was preincubated with 0 μM (lane 2), 5 μM (lane 3), 10 μM (lane 4) 20 μM (lane 5), 30 μM (lane 6), 40 μM (lane 7) 50 μM (lane 8) LPA (C18:1) before incubation with LPA beads. In lane 1 purified pyruvate kinase (1 μM) was incubated with control beads. Lanes 9-12: preincubation of 1 μM pyruvate kinase with 0 μM (lane 9), 50 μM (lane 10), 100 μM (lane 11) or 200 μM (lane 12) LPA (C6:0) before incubation with LPA beads. Retained pyruvate kinase was visualized by SDS-PAGE and coomassie staining.

Fig. 5. Effect of lysophosphatidic acid on pyruvate kinase activity.
A, 0.5 μM pyruvate kinase was incubated at room temperature with increasing concentrations of LPA (18:1) (■); LPA (14:0) (▲) and LPA (6:0)(♦) *B*, 0.5 μM pyruvate kinase was incubated at room temperature with increasing concentrations of LPA (18:1) (■); LPC (□); LPS (Δ) and LPI (▲). Activities are expressed relative to the activity of pyruvate kinase in buffer solution. None of these lysophospholipids has an effect on the LDH activity (data not shown). Symbols represent averages ± standard deviations from three experiments. *C*, Non-denaturing polyacrylamide gel electrophoresis of pyruvate kinase without LPA (lane 1), or with increasing concentrations of LPA (18:1) at pyruvate kinase/LPA molar ratios of 1/10, 1/20, 1/50, 1/100 and 1/200, respectively (lanes 2 to 6).

Fig. 6. Dissociation of pyruvate kinase tetramers by LPA. Elution profiles of pyruvate kinase on a Superdex 200 PG column are shown following 30 min pre-incubation with lysophospholipids at room temperature at a 1/100 molar ratio. Pyruvate kinase pre-incubated with buffer solution (○); LPA (18:1) (■); LPC (□); LPS (Δ) and LPI (▲). The fluorescence emission of tryptophane was used to monitor

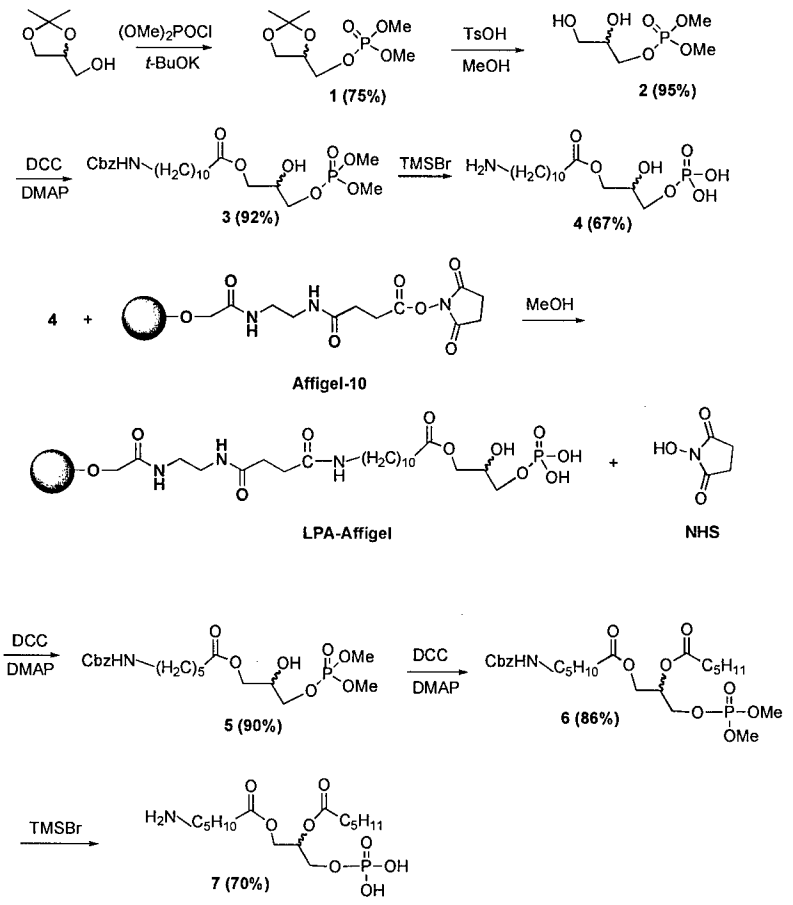
elution of the enzyme. The elution position of internal standards is indicated by arrows: catalase (1), lactate dehydrogenase (2), ovalbumin (3).

Fig. 7. LPA increases alfa helicity in pyruvate kinase. Circular dichroism spectra of pyruvate kinase are shown after 30 min incubation with lysophospholipids at room temperature at a 1/100 molar ratio. Pyruvate kinase in buffer solution (○); LPA (18:1) (■); LPC (□); LPS (Δ) and LPI (▲). Protein concentration was 0.1 mg/ml and the cuvette path length was 0.1 cm.

Fig. 8. Pyruvate kinase occurs in a complex with clathrin heavy chain and Hsp70. MCF-7 cell lysates were immunoprecipitated with antibodies against pyruvate kinase (left) or against clathrin heavy chain (right) (lane 1). The immunoprecipitate was analyzed for the presence of pyruvate kinase, Hsp70 or clathrin heavy chain by immunoblotting with specific antibodies. Lanes 2-4 represent controls : non-specific rabbit IgG (lane 2) or mouse IgG (lane 2); crude cell lysate (lane 3) and protein G-Sepharose without antibodies (lane 4).

Fig. 9. Co-localization of endogenous pyruvate kinase and clathrin. MCF-7 cells were fixed and stained for pyruvate kinase or clathrin heavy chain and analyzed by confocal microscopy (A) Staining of endogenous pyruvate kinase in MCF-7 cells (green). (B) Staining of endogenous clathrin heavy chain in MCF-7 cells (red). (C) Merged image. White arrows indicate co-localization between pyruvate kinase and clathrin heavy chain. Bar = 10 μm.

Figure 1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1 (75%)
TsOH
MeOH
2 (95%)
DCC
DMAP
3 (92%)
TMSBr
4 (67%)
4 +
MeOH
Affigel-10
LPA-Affigel
NHS
2
DCC
DMAP
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DCC
DMAP
6 (86%)
TMSBr
7 (70%)

Figure 2

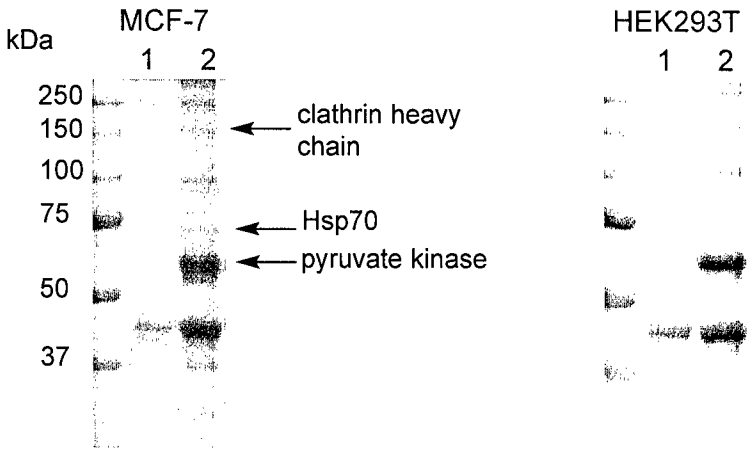
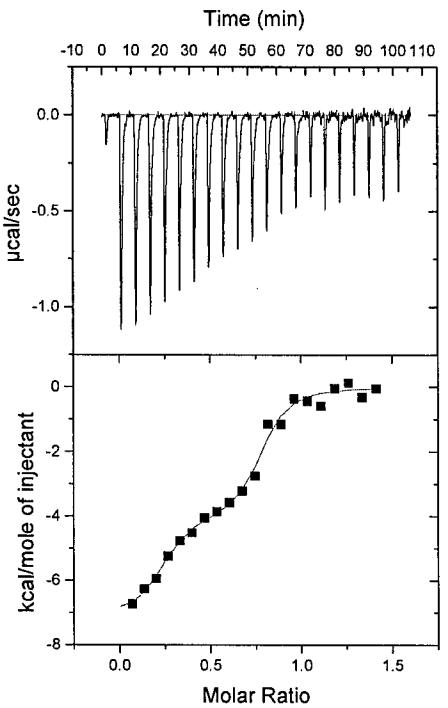


Figure 3



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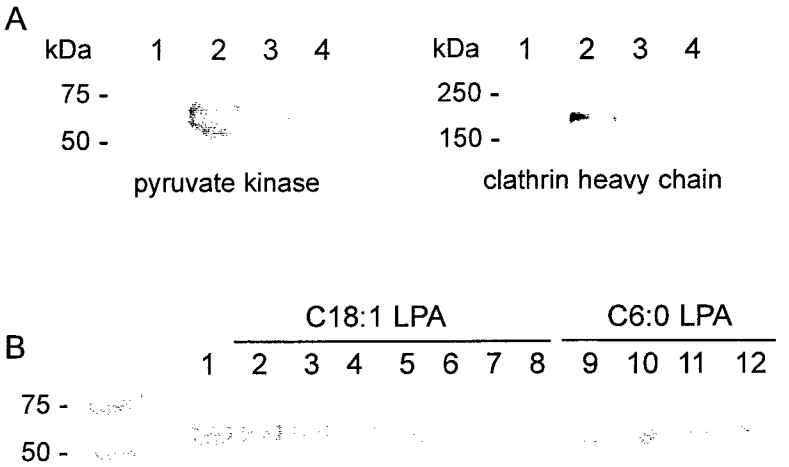
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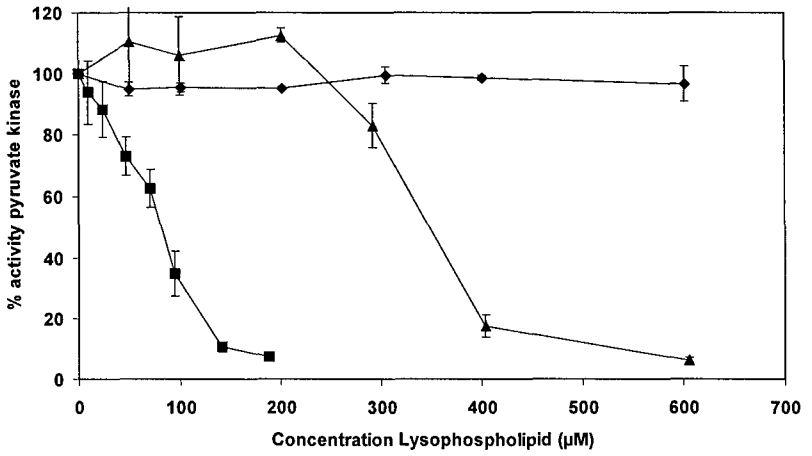
kDa 1 2 3 4
75 -
50 -
pyruvate kinase
kDa 1 2 3 4
250 -
150 -
clathrin heavy chain
B
C18:1 LPA
C6:0 LPA
1 2 3 4 5 6 7 8 9 10 11 12
75 -
50 -

Figure 5

A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100
80
60
40
20
0
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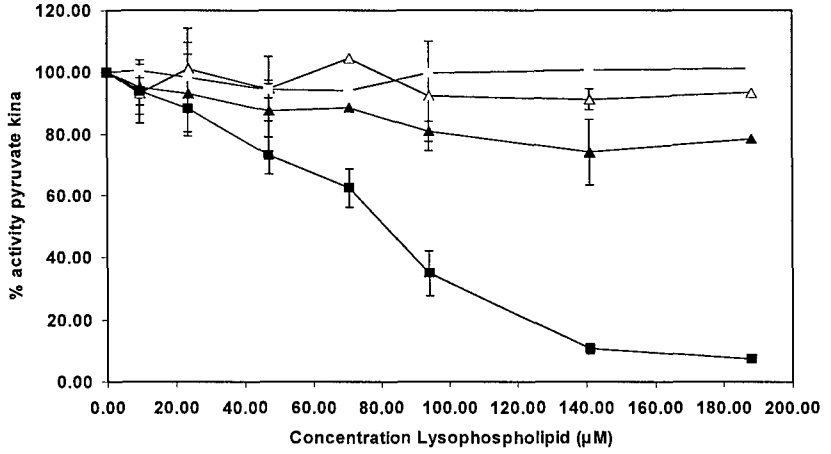
100
200
300
400
500
600
700
Concentration Lysophospholipid (µM)

B


120.00
100.00
80.00
60.00
40.00
20.00
0.00
% activity pyruvate kina
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20.00
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80.00
100.00
120.00
140.00
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180.00
200.00
Concentration Lysophospholipid (µM)

C

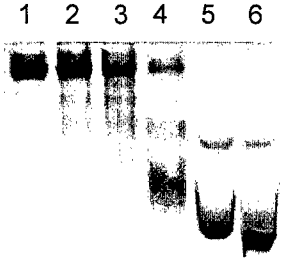

1
2
3
4
5
6

Figure 6

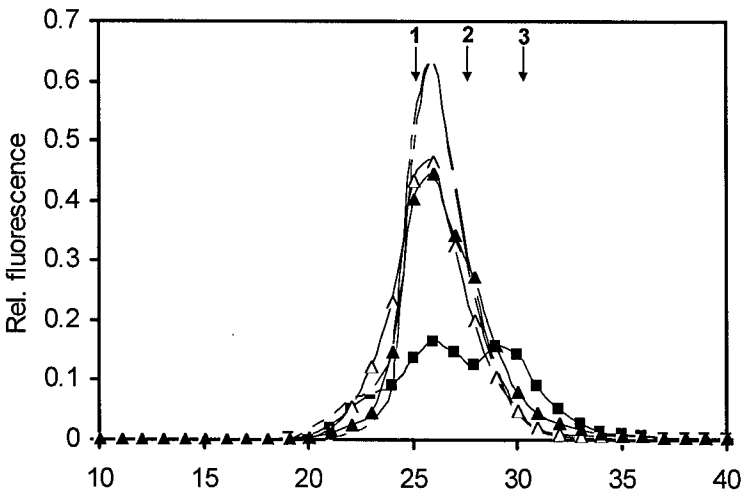


Figure 7

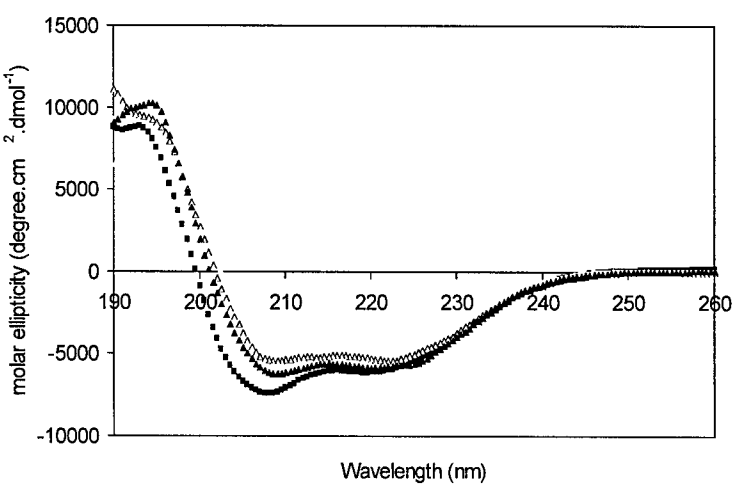


Figure 8

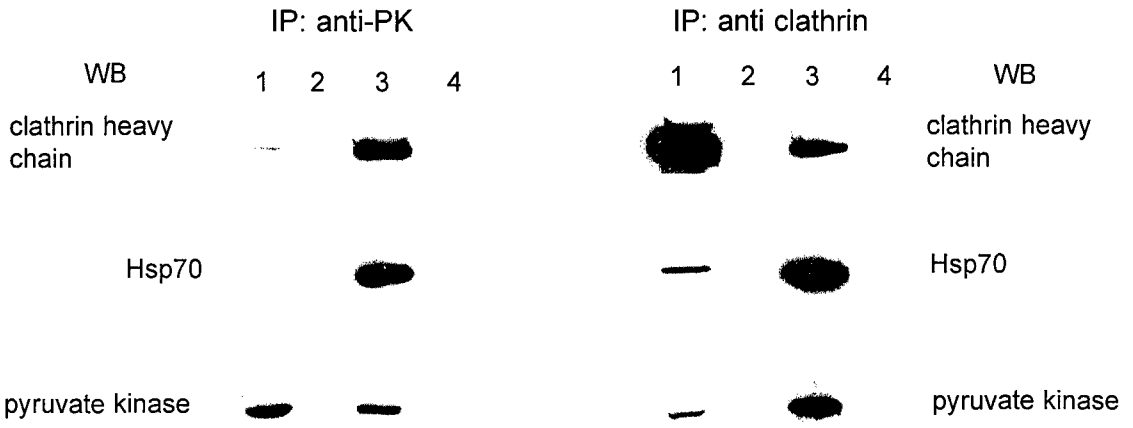
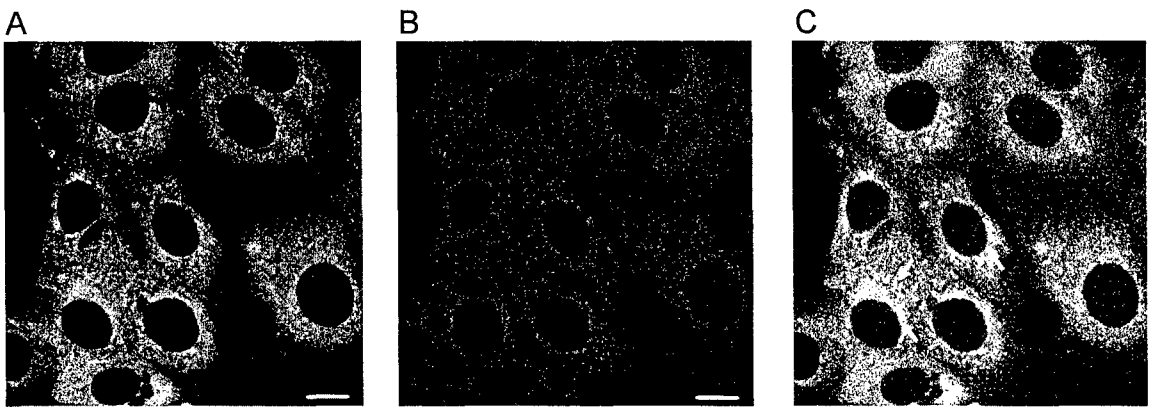


Figure 9



Lynn Guy
05/02/05 09:50 AM

To: Gordon Mills/MDACC@MDACC, Lisa Walker/MDACC@MDACC, Martha Matza/MDACC@MDACC, Wanda A. Quezada/MDACC@MDACC

cc:

Subject: DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry"

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

Institutional Review Board (IRB)
Box 574
Phone 713-792-2933
Fax 713-794-4589

Office of Protocol Research

To: Gordon Mills 05/02/2005
From: Lynn Guy
CC: Lisa Walker, Martha Matza, Wanda A. Quezada
MDACC Protocol ID #: LAB05-0139
Protocol Name: DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry"
Status: Activated

Subject: Activation and Distribution of Protocol Entitled "DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry""

This study is now active and ready for patient accrual.
Your signature page has been received and this study will be activated today.

The Informed Consent(s) will be available in the Informed Consent Printer Database within 30 minutes.

Sincerely,

Lynn Guy 05/02/2005 09:50:41 AM

DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry"
LAB05-0139

Subtitle: SELDI

Request for Waiver of Informed Consent

Protocol Number: **LAB05-0139**
Principal Investigator: **Gordon Mills**
Protocol Title: **DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry"**

The research involves no more than minimal risk to the subjects.
The samples will be obtained from existing tumor banks in breast, ovarian, and lung. These banks will have independent IRB approval for collection.

The waiver or alteration will not adversely affect the rights and welfare of the subjects.
The patient information will be provided in a confidential manner that cannot be linked to the patient by the investigator. The investigator will only receive "tumor bank number".

The research could not practicably be carried out without the waiver or alteration.
Many of the patients are deceased and cannot grant a waiver. Others cannot be contacted or traced, particularly international patients.

Whenever appropriate, the subjects will be provided with additional pertinent information after participation.
This is a retrospective study and the importance of the information will not be known until the data is analyzed and validated in a prospective study. No data will be referred on the basis of this retrospective study.

Waiver of Informed Consent Date:	04/26/2005
For IRB Use Only: Waiver Approved: Yes	Reviewer: Steven M. Kornblau
The IRB responsible for review and approval of this waiver was The University of Texas M. D. Anderson IRB #1 IRB00000121	

Waiver of Authorization to Use and Disclose Protected Health Information (PHI)

Protocol Number: LAB05-0139
Principal Investigator: Gordon Mills
Protocol Title: DAMD17-03-1-0222 "Detection of serum Lysophosphatidic Acids Using Affinity Binding and Surface Enhanced Laser Absorption/Ionization (SELDI) Time of Flight Mass Spectrometry"

1. **The use or disclosure of the PHI involves no more than minimal risk to the individual's privacy. This is based on the following 3 criteria:**

   (a) The research protocol includes adequate plans to protect identifiers from improper use.
   The samples will be provided from existing tumor banks. The investigator will only receive clinical information that cannot be linked to the patient and a tumor bank number.

   (b) The research protocol includes an adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research.
   The investigator will not receive identifiers that can be linked to the patient but will only receive tumor bank numbers. These and the patient clinical information will be destroyed at the completion of the study.

   (c) The research protocol includes adequate written assurances that the PHI will not be reused or disclosed to any other person or entity, or for other research.
   Will will only use the PHI as described specifically in the protocol. We will not reuse or disclose the information or use the information for other research.

2. **The research could not practicably be conducted without this waiver or alteration.**
   Many of the patients are deceased and cannot grant a waiver. Others cannot be contacted or traced, particularly international patients.

3. **The research could not practicably be conducted without access to and use of the PHI.**
   The research is aimed at determining whether particular alterations indicate prognosis. The patient PHI is required for this purpose.

Waiver of Authorization Date:	04/26/2005
Steven M. Kornblau	
Print Name of IRB Authorized Individual	Signature
This waiver was reviewed and approved by the following method:	○ Full Committee ◉ Expedited Review

The IRB responsible for review and approval of this waiver was The University of Texas M. D. Anderson IRB #1 IRB00000121